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Habilitationsschrift

Auf dem Weg zur personalisierten Medizin: Weiterentwicklung molekulargenetischer Techniken

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von

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Abkürzungsverzeichnis

ACE	Angiotensin Converting Enzym
AVI	Aortic Valve Implantation
AVR	Aortic Valve Replacement
BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
CEP	Chromosome Enumerator Probe
Chr	Chromosom
COPD	Chronic Obstructive Pulmonary Disease
CPM	Confined Placental Mosaicism
CRP	C-reaktives Protein
DAPI	4,6-diamino-2-phenylindole
DM	Diabetes Mellitus
DNA	Desoxyribose Nucleic Acid = Desoxyribonukleinsäure
et al.	et alii (und andere)
FisH	Fluoreszent-in-situ-Hybridisierung
HGP	Human Genome Project = Humanes Genom Projekt
iCTB	invasive Cytotrophoblast
IT	Information Technology = Informationstechnologie
LINE	Long Interspersed DNA Repeat
LSP	Locus-Specific Probe
MH	Maligne Hypertonie
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction = Polymerasekettenreaktion
RF	Renal Failure
SAB	Spontaneous Abortions
SD	Standard Deviation
SINE	Short Interspersed DNA Repeat
tAVI	transcatheter Aortic Valve Implantation
UCSC	University of California Santa Cruz
YAC	Yeast Artificial Chromosome

1. Einleitung

Mit Evidenz-basierter Medizin streben wir danach, die Ergebnisse robuster klinischer Studien heranzuziehen, um die optimale Therapie für jeden einzelnen unserer Patienten zu planen.
Ich unterstütze dieses Konzept vollauf und versuche, jeden Tag Evidenz-basierte Medizin zu praktizieren.
Ein bislang nicht zufriedenstellend gelöstes Problem liegt in der Übertragung der Ergebnisse großer, signifikanter Studien mit großen - und somit heterogenen - Patientenkohorten auf die Bedürfnisse eines individuellen Patienten in unserer Fürsorge.
Es ist mittlerweile Standard, bei großen Studien Subgruppen-Analysen vorzunehmen und die Übertragbarkeit oder Variabilität der Ergebnisse zum Beispiel nach Geschlecht, Komorbidität oder Risikofaktoren zu untersuchen.
Ich bin jedoch überzeugt, dass der nächste große Schritt bei der Optimierung individueller Therapien in der Analyse des genomischen Profils einzelner Patienten und der darauf eingehenden Anpassung unserer Interventionen liegt.
Den individuellen Risiken zu begegnen und die jeweiligen Chancen zu nutzen, ist das Ziel der individuell angepassten Medizin. Diese wird zumeist als 'personalisierte Medizin', nach dem englischen 'personalised medicine' benannt. Da der Begriff 'personalisierte Medizin' jedoch auch oft verwendet wird, um eine ganzheitliche Behandlung des Patienten zu beschreiben, haben sich zudem die Begriffe 'individualisierte' oder 'stratifizierte' Medizin etabliert [1].
Die molekulargenetische Grundsatzforschung hat Techniken hervorgebracht, die heute in der klinischen Onkologie regelmässig angewandt werden, um direkt die Optimierung der Therapiestrategie zu ermöglichen.
Die breite Anwendung genomischer Techniken zur Stratifizierung nicht-onkologischer Krankheitsbilder ist hingegen noch nicht gegeben.
Nichtsdestotrotz beobachten wir auch in unserem nicht-onkologischen klinischen Alltag täglich beeindruckende Beispiele von Variabilität zwischen unseren Patienten, selbst solchen mit nominell gleichen Krankheitsbildern. Diese Variabilität im Phenotyp begründet sich in Unterschieden entlang des Spektrums der Genotyp - Phenotyp Interaktion.

In dieser kumulativen Habilitationsschrift werden Ergebnisse zusammengeführt, die diese Variabilität verdeutlichen, um dann im Kern Beispiele aufzuzeigen, wie die konsequente Neu-und Weiterentwicklung genomischer und im Besonderen molekulargenetischer Techniken die Anwendbarkeit genetischer Typisierung im klinischen Alltag ermöglichen kann.

2. Eigene Arbeiten

Die erste hier angeführte Originalarbeit beschreibt ein klinisches Beispiel von Phenotyp-Variabilität zwischen Patienten: auf einen relativ standardisierten Stimulus in Form einer Operation oder Intervention reagieren Patienten mit signifikant unterschiedlichen Entzündungsreaktionen. Der Fokus der Arbeit liegt in dem Vergleich der Entzündungsreaktion in Abhängigkeit von zwei verschiedenen Operationsverfahren. Doch zudem werden Daten präsentiert, die eine deutliche Variabilität zwischen Patienten nach gleichartigen Eingriffen aufzeigen.

2.1. Originalarbeit:

Transapical or Transluminal Approach to Aortic Valve Implantation Does Not Attenuate Inflammatory Response [2]

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Zusammenfassung

Kardiopulmonaler Bypass und herzchirurgische Eingriffe bedingen eine Entzündungsreaktion. Diese kann unter anderem durch eine Erhöhung des C-reaktiven Proteins (CRP), einem unspezifischen Entzündungsmarker, quantifiziert werden.

Vorherige Publikationen haben typische Verläufe dieser CRP Erhöhung nach Aorto-coronaren Venen Bypässen und nach Aortenklappenersatz unter Einsatz der Herzlungenmaschine beschrieben.

Regressionsanalysen zur Untersuchung möglicher Faktoren, welche diese Reaktion modifizieren könnten haben gezeigt, daß chronische Erkrankungen, wie etwa chronische Herzinsuffizienz, Diabetes und Lungenerkrankungen, sowie Übergewichtigkeit und Geschlecht alle einen Einfluß auf die CRP Reaktion haben können.

In der Originalarbeit untersuchen wir die Entzündungsreaktion nach Aortenklappenimplantation durch Katheter-basierte interventionelle Techniken, vornehmlich über den transapikalen Zugang, aber auch über transfemorale und transaxilliäre Zugänge. Dies ist eine retrospektive 'Case-Control' Studie.

68 Patienten welche eine interventionelle Aortenklappenimplantation über einen transapikalen (59), transfemoralen (7) oder transaxillären Zugang erhielten, wurden mit 68 Patienten welche einen konventionellen Aortenklappenersatz unter Zuhilfenahme der Herzlungenmaschine erhielten, verglichen. Hierzu wurden die Patienten bezüglich der Faktoren Alter, Geschlecht, BMI und Vorliegen oder Abwesenheit von chronischen Erkrankungen (Diabetes, Lungenerkrankungen oder Niereninsuffizienz) einander zugeordnet. Wir verglichen die perioperativen CRP Konzentrationen, Euroscores, sowie Outcome Daten (Zeit bis Extubation und 30 Tage Mortalität) der beiden Gruppen.
Alle Daten wurden prospektive gesammelt und retrospektive verglichen.

Die beiden Gruppen, also die Kohorte mit interventioneller Aortenklappenimplantation und die Kohorte mit konventionellem Aortenklappenersatz, waren sich in allen gematchten Faktoren ähnlich: Alter, Geschlechtsverteilung, BMIs und chronische Nebenerkrankungen waren vergleichbar.

Wie zu erwarten, hatte die Gruppe mit interventioneller Aortenklappenimplantation einen signifikant höheren medianen Euroscore.

Die Zeitverläufe der CRP Profile waren ähnlich in beiden Gruppen.
Jedoch zeigte die Gruppe mit interventioneller Aortenklappenimplantation signifikant höhere CRP Spitzenwerte an den 2., 3. und 4. postoperativen Tagen.

Beatmungszeit und 30 Tage Mortalität, als Indikatoren des jurzfristigen Outcomes, waren in beiden Gruppen ähnlich.

Die Anwendung eines interventionellen Zugangs zum Ersatz der Aortenklappe, und somit die Vermeidung der Anlage einer Herzlungenmaschine und die Verminderung des chirurgischen Traumas, reduziert nicht die unspezifische Entzündungsreaktion des Patienten.

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Diese Originalarbeit wurde in die kumulativen Habilitationsschrift aufgenommen, da zusätzlich zu den vornehmlich untersuchten Unterschieden in der Entzündungsreaktion zwischen den beiden Gruppen auch interessante inter-individuell unterschiedliche Verläufe beschrieben werden.

So haben bei vergleichbarer Konstellation der die Entzündungsreaktion beeinflussenden Faktoren (chronische Nebenerkrankungen, Geschlecht, Übergewicht) und bei relativ standardisiertem chirurgischen Trauma einzelne Patienten deutlich unterschiedliche CRP Anstiege.

Diese Tatsache illustriert im Kontext dieser kumulativen Habilitationsschrift die großen inter-individuellen Unterschiede zwischen verschiedenen Patienten und ihre nicht sicher zu vorhersagende Reaktionen auf medizinische Interventionen.

Ich postuliere, daß diese im Wesentlichen auf die Verschiedenen genetischen Anlagen der Patienten zurückzuführen sind. Somit währen sie, bei besserem Verständnis der genotypischen Unterschiede, durchaus vorhersagbar.

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Die Beschreibung von Unterschieden im klinischen Verlauf ist nur der erste Schritt. Es gilt die physiologischen oder pathophysiologischen Gründe für diese Unterschiede des Phenotyps zu verstehen, um dann die Möglichkeiten der Modifikation dieser Abläufe zu erforschen und zum anderen, um Tests zu entwickeln, um die individuellen genetischen Voraussetzungen jedes einzelnen Patienten verstehen zu können.
Mit diesem ultimativen Schritt kann der Erkenntnisgewinn der Forschung in reellen Nutzen für den Patienten übersetzt werden. Das Ziel ist es, aus einem Portfolio von effektiven pharmakologischen und nicht-pharmakologischen Interventionen Evidenz-basiert genau die Therapiekombination definieren zu können, die diesem individuellen Patienten, um den wir uns gerade kümmern, optimal hilft.
Bei der Verfolgung diesen Ziels werden eine Vielzahl von Ansätzen und Techniken der Genforschung entwickelt und genutzt.
Die folgende Originalarbeit gibt eine Übersicht über den Stand dieses Forschungsgebietes.

2.2. Originalarbeit:

Current Genomics in Cardiovascular Medicine [3]

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Keywords: Cardiovascular disease, GWAS, Gene sequencing, Personalised medicine

Abstract

Cardiovascular disease (CVD) is a heterogeneous, complex trait that has a major impact on human morbidity and mortality. Common genetic variation may predispose to common forms of CVD in the community, and rare genetic conditions provide unique pathogenetic insights into these diseases. With the advent of the Human Genome Project and the genomic era, new tools and methodologies have revolutionised the field of genetic research in cardiovascular medicine. In this review, we describe the rationale for the current emphasis on large-scale genomic studies, elaborate on genome wide association studies and summarise the impact of genomics on clinical cardiovascular medicine and how this may eventually lead to new therapeutics and personalised medicine.

Introduction

Cardiovascular disease: CVD is a heterogeneous, complex trait that has a major impact on human morbidity and mortality. The field of cardiovascular medicine involves a broad spectrum of abnormalities that are characterised by various clinical and etiological features – the major categories being coronary artery disease, congenital heart disease, heart muscle disorders and conduction disorders. Despite tremendous progress in knowledge gained, CVD remains the leading cause of death in the United Kingdom [1] and it has overtaken infectious diseases as the leading cause of death worldwide [2]. Increased life expectancy has generated an unprecedented growth in the older segment of the population, with an accompanying rising burden of aging-associated cardiovascular disorders. CVD is heritable and a two generational history of CVD remains a major risk factor, therefore genetic studies have long been pursued to elucidate the underlying disease mechanisms. However, CVD is not a homogenous phenotype and its etiologic complexity has been a formidable challenge. Common genetic variation may predispose to common forms of CVD in the community, and rare

genetic conditions provide unique pathogenetic insights into these diseases. Public availability of vast amounts of detailed sequence information about the human genome, completed sequence data on other animal genomes, and private sector development of high-throughput genetic technologies has transformed in a few short years the conduct of cardiovascular genetics and genomics research from a primary focus on Mendelian disorders to a current emphasis on genome-wide association studies: GWAS.

However, the clinical practice of cardiovascular medicine is largely governed by a phenotype based approach. Nevertheless, functional genomics has led to an improvement of our understanding of CVD and can be translated to clinical utility. Gene-based pre-symptomatic prediction of illness, finer diagnostic sub-classifications and improved risk assessment tools will permit earlier and more targeted intervention. Pharmacogenetics will guide our therapeutic decisions and monitor response to therapy. Personalized medicine requires the integration of clinical information, stable and dynamic genomics and molecular phenotyping.
In this review, we describe the rationale for the current emphasis on large-scale genomic studies, elaborate on genome wide association studies and summarise the impact of genomics on clinical cardiovascular medicine and how this may eventually lead to new therapeutics and personalised medicine.

Genome Wide Association Studies (GWAS)

We have accepted that CVD is a complex genetic trait showing familiality but no precise mode of inheritance. However, in the "pregenome era" pedigree studies have provided evidence for Mendelian mode of transmission for rare familial CVD, ion channel dysfunction underlying long QT syndrome, and sarcomeric and cytoskeletal protein abnormalities underlying hypertrophic and dilated cardiomyopathies. However, these mutations only account for a minority of the genetic basis of cardiovascular diseases [2-4]. It is now possible to systematically search the entire human genome for common variants that are associated with a particular phenotype. This is largely due to the advances in large scale genotyping after the completion of the Human Genome Project in 2003 [5,6] and the Hap Map in 2005 [7,8]. But leveraging this wealth of genetic information relies on the principle of Linkage Disequilibrium: LD is used to identify a set of common variants in the human genome that are excellent statistical proxies for genetic variation at a particular frequency [9].
GWAS have become more popular in recent years. Their rise can be attributed to the availability of whole genome Single Nucleotide Polymorphism (SNP – variant DNA sequence due to change in a single nucleotide) panels (e.g. the Illumina 1 M and Affymetrix 6.0 arrays, each of which can scan >1 million SNPs), large-scale genotyping technologies that are available at multiple genetics centres, the rapidly dropping sequencing cost per genome and powerful association analysis methods and accompanying software [10,11].
GWAS are case-control studies, wherein research subjects are typed for a large number of SNPs, typically 300,000 to 10,000,000, and the allele or genotype frequencies are evaluated for differences between groups or for correlations with continuous traits. GWAS are primarily designed to provide an unbiased survey of the effects of common genetic variants. The power of the GWAS to detect the phenotype associated alleles depends directly on the sample size of the study population, MAF (Minor allele frequency i.e. the frequency at which the less common allele of the SNP occurs in the

population), strength of LD between the markers, the causal variants and the effect sizes of the alleles.
At present, 3 conditions must be satisfied to be considered susceptible loci through GWAS: (1) sufficient sample size in the genome-wide scan (at least 1000 each of cases and controls), (2) association *P*-value at the genome-wide significance level ($P< 5 \times 10^{-8}$) and (3) confirmation of the association by independent replication studies [12].

GWAS and Coronary artery disease

It has been estimated that heritable factors account for 30%–60% of the inter-individual variation in the risk of coronary artery disease: CAD [13]. Genome-wide association studies have identified several common variants that associate with risk of CAD [14]. Recently, a meta– analysis of 14 GWAS of coronary artery disease comprising 22,233 individuals with CAD (cases) and 64,762 controls of European descent was completed, followed by genotyping of top association signals in 56,682 additional individuals. This analysis identified 13 loci newly associated with CAD at $P < 5 \times 10^{-8}$ and confirmed the association of 10 of 12 previously reported CAD loci. The 13 new loci showed risk allele frequencies ranging from 0.13 to 0.91 and were associated with a 6% to 17% increase in the risk of CAD per allele [15]. A recent genome wide association analysis of 2078 CAD cases and 2953 control subjects, identified 950 single nucleotide polymorphisms: SNPs associated with CAD at $p < 10^{-3}$. Subsequent use of data on genetic variants and addition of data on global monocytic gene expression revealed a novel association signal at chromosome 10q 23.31 within the LIPA: lysosomal lipase acid A gene. An assessment of LIPA SNPs and transcript with cardiovascular phenotype revealed an association of LIPA transcript levels with impaired endothelial function [16].
The GWAS loci associated with CAD are shown in table 1.

Region	Reported Gene(s)	SNP	Risk allele	P	Ref	OMIM Ref	GWAS Identifier
1p32.3	PCSK9	rs11206510	T	9.10 ◊ 10-8	17	607786	HGVST11
1p13.3	SORT1	rs599839	A	2.89 ◊ 10-10	18	*602458	HGVST11
1q41	MIA3	rs17465637	C	1.36 ◊ 10-8	18	613455	HGVST148
2q33.1	WDR12	rs6725887	C	1.12 ◊ 10-9	17		
3q22.3	MRAS	rs2306374	C	3.34 ◊ 10-8	19	*608435	HGVST93
6p24.1	PHACTR1	rs12526453	C	1.15 ◊ 10-9	17	*608723	HGVST11
6q25.3	LPA	rs3798220	C	3.00 ◊ 10-11	20	152200	HGVST75
9p21.3	CDKN2A,	rs4977574	G	1.35 ◊ 10-22	18,21,22	*600160	HGVST11
	CDKN2B						
10q11.21	CXCL12	rs1746048	C	2.93 ◊ 10-10	18	600835	HGVST11
12q24.12	SH2B3	rs3184504	T	6.35 ◊ 10-6	23	605093	
19p13.2	LDLR	rs1122608	G	9.73 ◊ 10-10	17	606945	HGVST75
21q22.11	MRPS6	rs9982601	T	4.22 ◊ 10-10	17	*611973	
1p32.2	PPAP2B	rs17114036	A	3.81 ◊ 10-19	15	*607125	
6p21.31	ANKS1A	rs17609940	G	1.36 ◊ 10-8	15	*608994	
6q23.2	TCF21	rs12190287	C	1.07 ◊ 10-12	15	*603306	HGVST11
7q32.2	ZC3HC1	rs11556924	C	9.18 ◊ 10-18	15		
9q34.2	ABO	rs579459	C	4.08 ◊ 10-14	15	110300	HGVST11
10q24.32	CYP17A1,CNNM2	rs12413409	G	1.03 ◊ 10-9	15	*609300	HGVST11
	NT5C2					*607803, *600417	
11q23.3	ZNF259, APOA5-	rs964184	G	1.02 ◊ 10-17	15	*603901	HGVST11
	A4-C3-A1				15	*606368	
13q34	COL4A1	rs4773144	G	3.84 ◊ 10-9	15	*120130	HGVST75
	COL4A2					*120090	HGVST11
14q32.2	HHIPL1	rs2895811	C	1.14 ◊ 10-10	15		
15q25.1	ADAMTS7	rs3825807	A	1.07 ◊ 10-12	15	*605009	HGVST11
17p13.3	SMG6, SRR	rs216172	C	1.15 ◊ 10-9	15	*610963, *606477	HGVST11
17p11.2	RASD1, SMCR3,	rs12936587	G	4.45 ◊ 10-10	15	*605550	
	PEMT			1.81 ◊ 10-8	15	*602391	
17q21.32	UBE2Z, GIP,	rs46522	T			*137240	HGVST11
	ATP5G1, SNF8					*603192,*610904	HGVST11
10q23.31	LIPA	rs1412444	T	6.29 ◊ 10-4	16	*613497	HGVST11
		rs2246833	T	6.78 ◊ 10-4	16		

Table 1: GWAS Loci for CAD

GWAS and QT/QRS

The QT interval represents the time for both ventricular depolarisation and repolarisation to occur and hence estimates the duration of ventricular action potential. Long QT syndrome is due to prolonged repolarisation of the ventricular myocyte, manifest as a prolonged QT interval on the ECG that predisposes to ventricular tachycardia in the form of torsade des pointes, ventricular fibrillation and sudden cardiac death. Long QT syndrome is typically an isolated, autosomal dominant condition with significant variability in disease penetrance even in those harbouring the same disease-causing mutation, although the condition may be associated with sensorineural deafness and a more severe cardiac phenotype when inherited in a recessive fashion. However, the genetic mutations identified in the Mendelian form explain < 25% of the heritability.
QT-interval length on ECG was among the first CVD phenotypes to be studied by GWAS, revealing a strong association with SNPs in the *NOS1AP* gene (1q23.3) in a staged study that involved 2 independent cohorts and was subsequently independently replicated in another large cohort [24,25]. Unlike most genes known to be associated with QT-interval length, *NOS1AP* does not encode an ion channel but rather is a regulator of nitric oxide synthase; its mechanism of prolonging the QT interval is unknown. Moreover, 9 additional loci along-with NOS1AP have been associated with QT duration by the QTSCD and QTGEN consortium [26, 27], which has led to the identification of new candidate genes with ventricular arrhythmias and sudden cardiac death.

The electrocardiographic QRS interval reflects ventricular depolarization, and its duration is a function of electrophysiological properties within the His-Purkinje system and the ventricular myocardium. A diseased ventricular conduction system can lead to life-threatening brady-arrhythmias, such as heart block, and death in the general population and in cohorts with hypertension and coronary artery disease [28, 29, 30]. In a population-based study, prolonged baseline QRS was associated with incident heart failure. A significant association between QRS duration and risk of congestive cardiac failure was observed. Incomplete and complete bundle branch block were associated with a 1.5- and 2- fold risk of congestive cardiac failure respectively [31].

Twin and family studies suggest a genetic contribution to QRS duration, with heritability estimates of up to 40% [32, 33]. Candidate gene and genome-wide studies identified a limited number of loci associated with QRS duration, supporting the hypothesis of the contribution of common genetic variation in QRS duration [34, 35, 36].

A recent meta-analysis of 14GWAS consisting of 40,407 individuals of European descent with additional genotyping in 7170 Europeans yielded genome wide significance associations of QRS duration with common variants in 22 loci. Variation in four of these loci (locus 1, *SCN5A-SCN10A*; locus 2, *CDKN1A;* locus 8, *TBX5;* and locus 21, *DKK1*) were previously associated with QRS duration in smaller independent studies using both candidate gene and genome-wide approaches [34 - 36].The 22 loci include genes in a number of interconnected pathways, including some previously known to be involved in cardiac conduction, such as sodium channels, calcium-handling proteins and transcription factors, as well as previously unidentified processes not known to be involved in cardiac electrophysiology, such as kinase inhibitors, growth factor-related genes and others. They also demonstrated that *SCN10A*, a candidate gene at the most significantly associated locus in this study, is expressed in the mouse ventricular conduction system, and treatment with a selective *SCN10A* blocker prolongs QRS duration. GWAS loci associated with QRS duration are illustrated in table 2.

Locus	Chromosome	Reported Gene(s)	SNP	Risk allele	P	Ref	OMIM	GWAS Identifier
1	3	SCN10A	rs6801957	T/C	1.10 ◊ 10-28	37	*604427	HGVST489
	3	SCN10A-SCN5A	rs9851724	C/T	1.91 ◊ 10-20	37	*600163	HGVST489
	3	SCN5A/EXOG	rs10865879	T/C	1.10 ◊ 10-28	37	*604051	
	3	SCN5A	rs11710077	T/A	5.74 ◊ 10-22	37	*600163	HGVST489
	3	SCN5A	rs11708996	C/G	1.26 ◊ 10-16	37	*600163	HGVST489
	3	EXOG	rs2051211	G/A	1.57 ◊ 10-8	37	*604051	
2	6	CDK1NA	rs9470361	A/G	3.00 ◊ 10-27	37		
3	6	C6orf204-SLC35F1	rs11153730	C/T	1.26 ◊ 10-18	37		
		PLN-BRD7P3				37	*172405	
4	1	NFIA	rs9436640	G/T	4.57 ◊ 10-18	37	*600727	HGVST658
5	5	HAND1-SAP3OL	rs13165478	A/G	7.36 ◊ 10-14	37	*602406	HGVST658
6	7	TBX20	rs1362212	A/G	1.12 ◊ 10-13	37	*606061	
7	14	SIPA1L1	rs11848785	G/A	1.04 ◊ 10-10	37		
8	12	TBX5	rs883079	C/T	1.33 ◊ 10-10	37	*601620	HGVST489
9	12	TBX3	rs10850409	A/G	3.06 ◊ 10-10	37	*601621	
10	10	VTI1A	rs7342028	T/G	4.95 ◊ 10-10	37		
11	18	SETBP1	rs991014	T/C	6.20 ◊ 10-10	37	*611060	
12	2	HEATR5B-STRN	rs17020136	C/T	1.90 ◊ 10-9	37		
13	3	TKT-PRKCD-CACNA1D	rs4687718	A/G	6.25 ◊ 10-9	37	*606781	
14	2	CRIM1	rs7562790	G/T	8.22 ◊ 10-9	37	*606189	
15	1	C1orf185-RNF11	rs17391905	G/T	3.26 ◊ 10-10	37	*612598	
		CDKN2C-FAF1				37	*603369	
16	17	PRKCA	rs9912468	G/C	1.06 ◊ 10-8	37	*176960	HGVST658
17	7	IGFBP3	rs7784776	G/A	1.28 ◊ 10-9	37	*146732	
18	1	CASQ2	rs4074536	C/T	2.36 ◊ 10-8	37	*114251	HGVST658
19	13	KLF12	rs1886512	A/T	1.27 ◊ 10-8	37	*607531	HGVST658
20	3	LRIG1-SLC25A26	rs2242285	A/G	1.09 ◊ 10-8	37	*611037	
21	10	DKK1	rs1733724	A/G	3.05 ◊ 10-8	37	*605189	
22	17	GOSR2	rs17608766	C/T	4.75 ◊ 10-10	37	*604027	HGVST658

Table 2: GWAS Loci for QRS duration

It has also been shown in previous studies that in addition to their association with QRS duration, variants in *SCN5A* and *SCN10A* are associated with atrial conduction (PR interval) as well as atrial and ventricular fibrillation [35,36,38]. These results emphasize the crucial role played by these genes in cardiac conduction and the generation of arrhythmias.

GWAS and stroke susceptibility

Stroke is a leading cause of morbidity and mortality in the developed world. It can be broadly classified into ischaemic (cardio-embolic, large artery atherosclerosis or small vessel disease) and haemorrhagic stroke; with ischaemic stroke responsible for >80% of the cerebrovascular events.
In the first successful GWAS for ischemic stroke [39] the only signal to meet genome-wide significance was a SNP near PITX2 (paired-like homeodomain transcription factor 2), which had previously been associated with atrial fibrillation: AF [40]. AF, which is characterised by chaotic electrical activity of the atria, is one of the most common forms of electrical instability and is thought to be responsible for the majority of CES (cardio-embolic stroke) events [41]. Intriguingly, while the association was strongest for CES, the SNP was also associated with non-cardiogenic stroke, suggesting that this is due to a misclassification of the cause of stroke as a result of undiagnosed AF. Given the higher risk of recurrence in patients with CES, and greater morbidity and mortality, this might

have important clinical implications [42] .Two additional GWAS for stroke have been published, one focused on intracranial aneurysm [43], a major cause of haemorrhagic stroke, the other examined all strokes [42].

Disease	Region	Reported Gene(s)	SNP	Risk allele	Ref	OMIM Ref	GWAS Identifier
All stroke	12p13.33	NINJ2	rs12425791	A	44	*607297	HGVST302
Intracranial aneurysm	2q33.1	BOLL, PLCL1	rs700651	G	44	*606165,*600597	HGVST98
Intracranial aneurysm	8q11.23	SOX17	rs10958409	A	44	*610928	HGVST14
			rs9298506	A	44		
Ischaemic Stroke	9p21.3	CDKN2A, CDKN2B	rs1333040	T	44	*600160	HGVST14
Ischaemic Stroke	4q25	PITX2	rs220073	T	44	*601542	HGVST14

Table 3: GWAS Loci for Stroke

Bilguvar and colleagues [42] identified three loci associated with intracranial aneurysm, including the CDKN2A CDKN2B locus, which have previously been implicated in CAD and intracranial aneurysm (described above). Of the two novel loci identified, one (located on Ch 2q) is in a region containing four genes, and thus the relevant gene is unclear. The second locus (on Ch 8q) is particularly intriguing, as there seem to be two independent association signals flanking a single gene, SOX17 (SRY-box 17). The idea that associated genes are likely to harbour multiple independent variants is gaining support [45, 46] and suggests that novel analytical tools that account for this effect can increase power to identify genes through GWAS. The authors note that SOX17 is of particular interest because it is required for endothelial formation and maintenance, and Sox17-/- mice show vascular abnormalities [47]. In the second study, Ikram and colleagues identified a single locus, NINJ2, associated with stroke [42]. NINJ2 encodes ninjurin2, an adhesion molecule that is up- regulated in response to nerve injury. Analyses stratified by specific stroke subtype indicate that this locus is specific for ischemic stroke, which accounts for more than 2/3 of stroke cases.

GWAS and CVD risk factors

Given the complex aetiology of CVD, the study of cardiovascular risk factors is likely to facilitate the elucidation of cardiovascular risk. Association studies have targeted multiple unrelated phenotypes measured in epidemiological surveys. Given that effect-sizes for variants identified through GWAS were smaller than previously suspected, many groups have now combined GWAS scans to identify novel loci for these quantitative traits [23, 40, 48-52].
In two co-published papers, analysis of lipid concentrations in a combined total of 8 816 individuals [50] with follow-up of >10 000 samples in each study, identified 19 loci associated with various lipid traits. More recently, two GWAS for lipid levels, each with >20 000 individuals, have lead to the identification of 35 total loci influencing lipid levels [51]. Kathiresan and colleagues reported a total of 30 loci influencing LDLC, HDL-C and/or TG. They detected multiple independent hits at some of the loci, resulting in 37 independent signals; the proportion of explained variance in each trait was 9.3% for HDL-C, 7.7% for LDL-C, and 7.4% for TG. In addition to LDL-C, HDL-C, and TG, Aulchenko and colleagues [51] looked for association with total cholesterol (TC). Overall, they identified 22 loci explaining up to 4.8% of the variance in each individual lipid trait (4.8% HDL-C, 3.4% LDL-C, 3.0% TG). Using prospective data, Aulchenko and colleagues evaluated the clinical relevance of genetic ‘risk profiles’, in particular, the TC risk profile, and demonstrated an improvement in CHD risk classification beyond traditional clinical factors of lipids, age, BMI, and sex. In a

GWAS study of 100184 individuals of European ancestry (typed for 2.6 million SNP), 22 loci were associated with plasma LDL-C, 31 loci were associated with high-density lipoprotein cholesterol, and 16 loci were associated with triglycerides.These variants accounted for >25% to 30% of the genetic variance for each trait [53].

To date, GWAS have identified nearly 40 susceptibility loci for type 2 diabetes: T2D in European and Asian populations. The first GWAS for T2D was conducted in a French cohort composed of 661 cases and 614 controls, covering 392,935 SNP loci. This study identified novel association signals at *SLC30A8*, *HHEX*, *LOC387761*, conand *EXT2* and validated the previously identified association at *TCF7L2*[54].

The first round of European GWAS confirmed 8 T2D susceptibility loci across multiple ethnic groups: *TCF7L2*, *SLC30A8*, *HHEX*, *CDKAL1*, *IGF2BP2*, *CDKN2A*/*B*, *PPARG*, and *KCNJ11*. In addition to these 8 loci, the WTCCC/UKT2D study identified a strong association between *FTO* variants and T2D, although the effect of *FTO* variants on conferring susceptibility to T2D was mostly mediated through increase in body weight [55]. Most of the T2D genetics cohorts have now combined to form DIAGRAM+, which yields an effective sample size of more than 22,000 subjects of European origin. In a recent study, 2,426,886 imputed and genotyped autosomal SNPs, with additional interrogation of the X-chromosome, were examined for association with T2D as a categorical phenotype. Twelve new loci were identified as susceptibility loci for T2D with a genome wide significance association ($P < 5 \times 10\text{-}8$) [56].

A GWAS of systolic and diastolic blood pressure, which used a multi-stage design in 200,000 individuals of European descent identified 16 novel loci: six of these contain genes previously known or suspected to regulate blood pressure (GUCY1A3-GUCY1B3, NPR3-C5orf23, ADM, FURIN-FES, GOSR2, GNAS-EDN3) and the other ten provide new clues to blood pressure physiology [57].

Chromosome	Reported Gene	SNP	Risk Allele	P	Ref	OMIM	GWAS Identifier
1	MOV10	rs2932538	G/A	2.9 ◊ 10-7	57	*610742	HGVST9
3	SLC4A7	rs13082711	T/C	3.6 ◊ 10-4	57	*603353	HGVST9
3	MECOM	rs419076	T/C	3.1 ◊ 10-4	57		
4	SLC39A8	rs13107325	T/C	4.9 ◊ 10-7	57	*608732	
4	GUCY1A3–	rs13139571	C/A	2.5 ◊ 10-5	57	*139396	HGVST9
	GUCY1B3					*139397	HGVST9
5	NPR3–	rs1173771	G/A	3.2 ◊ 10-10	57	*108962	HGVST9
	C5orf23						
5	EBF1	rs11953630	T/C	1.7 ◊ 10-7	57	*164343	HGVST9
6	HFE	rs1799945	G/C	1.8 ◊ 10-10	57	*613609	HGVST9
6	BAT2–BAT5	rs805303	G/A	1.1 ◊ 10-10	57	*142620	
10	CACNB2(59)	rs4373814	G/C	8.5 ◊ 10-8	57	*600003	HGVST9
10	PLCE1	rs932764	G/A	3.2 ◊ 10-10	57	*608414	HGVST9
11	ADM	rs7129220	G/A	9.4 ◊ 10-9	57	*103275	HGVST9
11	FLJ32810–	rs633185	G/C	1.1 ◊ 10-3	57		
	TMEM133						
15	FURIN–FES	rs2521501	T/A	5.4 ◊ 10-11	57	*190030	HGVST9
17	GOSR2	rs17608766	T/C	7.0 ◊ 10-7	57	*604027	HGVST9
20	JAG1	rs1327235	G/A	4.6 ◊ 10-4	57	*601920	HGVST9
20	GNAS–EDN3	rs6015450	G/A	4.2 ◊ 10-14	57	*139320	HGVST9
1	MTHFR–	rs17367504	G/A	2.3 ◊ 10-10	57	*607093	HGVST9
	NPPB						
3	ULK4	rs3774372	T/C	0.18	57		
4	FGF5	rs1458038	T/C	1.9 ◊ 10-7	57	*165190	HGVST9
10	CACNB2(39)	rs1813353	T/C	6.2 ◊ 10-10	57	*600003	HGVST9
10	C10orf107	rs4590817	G/C	9.8 ◊ 10-9	57		
10	CYP17A1–	rs11191548	T/C	1.4 ◊ 10-5	57	*609300	HGVST9
	NT5C2						
11	PLEKHA7	rs381815	T/C	3.4 ◊ 10-6	57	*612686	HGVST9
12	ATP2B1	rs17249754	G/A	1.1 ◊ 10-14	57	*108731	HGVST9
12	SH2B3	rs3184504	T/C	2.6 ◊ 10-6	57	*605093	
12	TBX5–TBX3	rs10850411	T/C	5.2 ◊ 10-6	57	*601620	HGVST9
15	CYP1A1–	rs1378942	C/A	1.0 ◊ 10-8	57	*108330	HGVST9
	ULK3						
17	ZNF652	rs12940887	T/C	1.2 ◊ 10-7	57	*613907	HGVST9

Table 4: GWAS loci for BP

GWAS and aortic aneurysm

Thoracic aortic aneurysms and dissections: TAAD can be inherited as a single gene disorder, but the genetic predisposition in majority of the people is poorly understood. A recent multi-staged GWAS, compared 765 individuals with sporadic TAAD with 874 controls. The study identified common SNPs at 15q21.1 locus that associated with sporadic TAAD and attained genome wide significance. 107 SNPs associated with sporadic TAAD with $p < 1x\ 10^{-5}$ were then followed up in two separate sporadic TAAD cohorts.The associated SNPs were found in a large region in linkage disequilibrium encompassing FBN1, which encodes fibrillin. FBN1 mutations cause Marfan syndrome, whose major cardiovascular complication is sporadic TAAD. The study suggested that common genetic variants at 15q 21.1, (that probably act via FBN1) are associated with sporadic TAAD, suggesting a common pathogenesis of aortic disease in Marfan syndrome and sporadic TAAD [58].

Chromosome	Reported Gene	SNP	Risk allele	P	Reference	OMIM
15q21.1	FBN1	rs10519177	G	2.6 ◊ 10-11	58	*134797
15q21.1	FBN1	rs4774517	A	3.8 ◊ 10-11	58	*134797
15q21.1	FBN1	rs755251	G	3.2 ◊ 10-11	58	*134797
15q21.1	FBN1	rs1036477	G	6.5 ◊ 10-12	58	*134797
15q21.1	FBN1	rs2118181	G	5.9 ◊ 10-12	58	*134797

Table 5: GWAS loci for TAAD

GWAS: Limitations

Although GWAS have been very useful in identifying a large number of phenotype associated alleles, that may lead to novel pathways involved in the pathogenesis of the phenotype, they do have some rather important limitations.
The results of GWAS are subject to multiple-hypothesis testing. This is because a very large number of SNPs are typed to look for possible associations and hence this needs to be corrected for the possibility of random associations in statistical analysis. In addition, often the same study population is analysed for the association of the genotypes with multiple phenotypes, which also increases the likelihood of spurious associations.
Despite the very large number of phenotype- associated alleles identified by GWAS, the number of functional SNP associated alleles is scarce [59]. Hence the results have not been useful in the immediate elucidation of the responsible mechanisms behind the observed genetic association. Therefore, GWAS need to be complemented with mechanistic studies to unravel the biological mechanisms responsible for genetic association, rather than simply increasing sample size to increase the power of the study.
Alleles identified in GWAS are often not the true causative alleles but are likely in linkage disequilibrium with the true causative alleles. The precise identification of gene and causal variants in complex diseases is difficult. The most challenging aspect is that most associated SNPs map to non-coding segments of the genome; although these regions contribute to complex diseases they contain genetic motifs that we do not fully recognize, and thus do not necessarily identify a specific gene [60]. Targeted re-sequencing of the region in linkage disequilibrium to locate the region of true genetic variation is required. Thus, extensive additional studies are typically required to complement the results of GWAS to identify the disease causing alleles.
The results of GWAS have minimal to modest impact, if any, on preclinical diagnosis, risk stratification, or genetic based prevention and treatment at an individual level. This could be due to large number of common variants with low magnitude of effect, rare variants with large effects, interaction between alleles at homologous loci and between alleles at non-homologous loci, epigenetic effects and underestimation of the effect of shared environment among relatives leading to inflated estimation of heritability [61].
Also, there is a need to integrate data at multiple levels i.e. genetic variations along-with epigenetic profiles. This approach will become more attainable with the advent of "3rd Generation" sequencing technology (see below).

Gene Sequencing

By their very nature, GWAS focus on a very small percentage of the total genome, thus there is great potential to miss the causative variations, irrespective of whether these are in coding or non-coding regions. However, capturing all possible variation within a sample requires a sequencing strategy.

Current technologies

Since first introduced to the market in 2005, next-generation sequencing technologies have had a tremendous impact on genomic research. The next-generation technologies have been used for standard sequencing applications, such as genome sequencing and re-sequencing, and for novel applications previously unexplored by Sanger sequencing. The landmark publications of the late 1970 s by Sanger's and Gilbert's groups and notably the development of the chain termination method by Sanger and colleagues [62] established the groundwork for decades of sequence-driven research that followed. The chain-termination method published in 1977, also commonly referred to as Sanger or dideoxy sequencing, has remained the most commonly used DNA sequencing technique to date and was used to complete human genome sequencing initiatives lead by the International Human Genome Sequencing Consortium and Celera Genomics [63,64]. Very recently, the Sanger method has been partially supplanted by several “next-generation” sequencing technologies that offer dramatic increases in cost-effective sequence throughput, albeit at the expense of read lengths. The next-generation technologies commercially available today include the 454 GS20 pyro sequencing based instrument (Roche Applied Science), HiSeq (Illumina, Inc.), the SOLiD instrument from Applied Biosystems, and the Heliscope from Helicos, Inc. The read lengths of these technologies are 200-300 bp, 30-40 bp, 35bp and up to 900bp respectively.
Current “Next-generation” or “2nd Generation” sequencing essentially relies on a modified “shotgun” approach, involving multiple steps: template fragmentation and amplification, sequencing, imaging and alignment of the sequences to a reference genome. A key aspect is that current technology is unable to sequence much more that 1000 base pairs in a single reaction; indeed much of the published data is derived from very short sequencing “reads” of around 35bp, something that can cause problems with downstream analysis, as these short reads may align to multiple locations in the reference genome requiring computationally expensive alignment algorithms. Artefacts can also be introduced at various steps, particularly during PCR amplification of the template fragments. If personalized genomics is to be realised we also need to ensure that protocols are standardised to minimise the run-to-run, and lab-to-lab variation that has been reported [65].

Future Directions

Genome Enrichment: In the short to medium term, research groups with a finite budget have to make the decision whether to perform whole genome-sequencing on a small number of subjects, or exome-sequencing on a larger number, weighing up the relative merits of each along the way. Use of exome-seq has recently been supported by a report that the entire CCDS (a database of consensus coding sequences in human and mouse) exome can be interrogated with just 2.8 Gbp of sequence data, approximately 3% of the required data for whole genome shotgun experiments [66] (although this still does not

mitigate against the inability to identify variation in regulatory elements in non-coding regions).

3 rd Generation Technology: Second generation technology continues to evolve, with the main players continually improving both the hardware, and the chemistry that underpins sample preparation and the actually sequencing reaction itself. But already there are a number of technologies (the so-called 3rd Generation sequencers) that may well deliver on the promise to provide the $1000 genome. Single-molecule, real-time sequencing negates the need for amplification, removing one of the key sources of bias mentioned above. Companies such as Helicos and Pacific Biosciences [67-69] are leading the way. In addition to the single-molecule nature of these technologies, read length is significantly increased, improving the ability of the analytical software to successfully map the reads back to the reference genome. Finally, single molecule sequencing can detect methylation at single-base resolution allowing the integration of genetic and epigenetic data [70].

Perspective: Clinical Impact

Phenotype based approaches largely govern the current practice of cardiovascular medicine. The influence of genetic variants is expected to correlate inversely with the proximity of the phenotype to the genes. Genetic determinants of the proximal (biochemical) phenotypes could help unravel the biological and functional sequence of DNA coding variants and might be translated to the clinical phenotype in the future. Whole genome sequencing is likely to become readily available at a reasonable cost and the genome/exome data could link genotype to the phenotype prospectively. The challenge would be to differentiate the associated alleles from the disease causing variants by means of genetic and biological studies.
Despite the very large number of functional alleles identified by GWAS and the rapidly evolving field of functional genomics, their application in clinical cardiology are limited at present. However, these approaches have lead to an improvement of our understanding of CVD and can be translated to clinical utility. Gene-based pre-symptomatic prediction of illness, finer diagnostic sub-classifications and improved risk assessment tools will permit earlier and more targeted intervention. Pharmacogenetics will guide our therapeutic decisions and monitor response to therapy. Personalized medicine will require the integration of clinical information, stable and dynamic genomics, and molecular phenotyping.
These have already found a place in cardiovascular screening, diagnosis and pharmacogenesis. As recently reviewed by Kim et al, disease-causing mutations for a wide range of Mendelian cardiac disorders have been revealed through linkage studies, thereby permitting screening of family members to identify mutation carriers for early intervention [71]. A more controversial task is the use of common genetic variants identified in genome-wide association studies for risk prediction in the primary prevention of cardiovascular disease. Risk prediction using genomic information is still developing and the various predictors identified in GWAS are potential genomic predictors to cardiovascular risk. This is likely to improve in the future by inclusion of rare DNA variants identified by sequencing and incorporating functional genomic data into predictive models including epigenetic markers, transcriptomics and metabolomic biomarkers [72].
Non-invasive alternatives that reduce the need for invasive testing are a lucrative goal in clinical cardiology. Already in clinical use is the non-invasive diagnosis of cardiac

allograft rejection with blood gene expression. The Invasive Monitoring Attenuation through Gene Expression: IMAGE trial compared the routine use of endomyocardial biopsies for monitoring rejection with a more selective use of endomyocardial biopsy guided by a gene-expression profiling test called AlloMap and noninvasive cardiac imaging. Both strategies resulted in equivalent clinical outcomes, but patients who were monitored with gene-expression profiling underwent far fewer biopsies per person-year of follow-up than did patients who were monitored with routine biopsy (0.5 versus 3.0; P=0.001) [73].

Pharmacogenomics is the study of how genetic variation affects the clinical response to drugs, with the implicit assumption that pharmacogenomic insights will enhance efficacy and reduce toxicity. It is likely to be the field that will benefit the most in the short term as it is easy to link genetics to the ability to metabolise a drug. Recognizing that 25% of patients have a sub-therapeutic antiplatelet response to clopidogrel, researchers have identified several genetic variants affecting the metabolism of clopidogrel, a prodrug, to its active metabolite. Of these, the CYP2C19 variant allele has been best linked to impaired clopidogrel metabolism, reduced platelet inhibition, and a higher risk of adverse cardiovascular events after percutaneous coronary interventions [74]. Because of the cumulative data, the Food and Drug Administration has now altered the prescribing information for clopidogrel based on CYP2C19 genotype, a move that foreshadows the development of companion diagnostic testing and alternative inhibitors of ADP-mediated platelet activation that do not require metabolism by CYP2C19 [74]. In heart failure therapeutics, pharmacodynamic studies and post hoc analyses from clinical trials indicate that polymorphisms in the β1-adrenergic receptor affect the clinical actions of β-blockers [75, 76] whereas more limited data suggest common variants that affect therapeutic response in heart failure [77] and hyperlipidemia [78-80]. Warfarin is a widely used oral anticoagulant with several important indications, significant risks associated with either under-dosing or overdosing, and great patient-to-patient variability in dosing. Although many clinical and environmental factors are known to affect warfarin dosing,[81] several studies have identified common sequence variants in at least 2 genes (CYP2C9 and VKORC1) that strongly affect the pharmacokinetics and pharmacodynamics of warfarin.[82-84] Small clinical trials suggest that that genetically based warfarin-dosing algorithms may enhance the efficiency and safety of warfarin dosing[85,86].

Hence, clinical applications of genomics are already enhancing the practice of cardiovascular medicine. Advances in functional genomics will be increasingly important to practicing cardiovascular specialists in the coming years.

Personalised Medicine/Genomics

Although the driving force behind GWASs was originally the discovery of novel biological pathways through the use of scalable genome technology and its capacity for generating hypothesis-independent genetic associations, the results have generated much excitement about the potential clinical applications of these genetic markers for disease prediction, prevention and diagnosis. It is hoped that genomic discoveries would lead to personalised medicine, whereby healthcare interventions would be guided based on individual's genomic make up. Direct-to-consumer genetic testing companies now offer mutation analysis and SNP microarray sequencing based on the findings of traditional genetic research and GWASs before the clinical validity or utility of population screening. The challenge will be to reconcile people's concerns about genomic privacy and security with the need to allow researchers and clinicians data

access. Other implications including life/health insurance, psychological risks to the patients if they know they have a genetic variant (that means a significant increase in risk) and family screening have to be considered as well. Conversely, knowing early in life of the predisposition to certain conditions, could lead to lifestyle modification, risk prevention and early intervention.
Personalised medicine within cardiovascular medicine, has been emphasised in risk prediction models, especially coronary artery disease, where the goal is to identify individuals at risk so that early intervention and lifestyle modification can be initiated. Genotype based risk prediction is fixed from birth, allows early risk prediction, is less susceptible to biological variation over life and is easy to obtain with minimal measurement error. A series of recent studies attempted to demonstrate predictive utility of the 9p21 risk allele [87-96]. All showed significant association between the risk alleles and incident events, but only one showed any improvement in the C-statistic based on traditional risk factors [89].
As we discern more genomic markers–SNPs, copy number variants, and rare alleles– that influence the development and course of cardiovascular disease, our goal as evidence-based clinicians will be to apply this knowledge judiciously and responsibly for more personalized and cost-effective care.
Currently, there is an expanding gap between the availability of direct-to-consumer whole genome testing and physician knowledge regarding interpretation of test results. Advances in the genomic literacy of health care providers will be necessary for genomics to fulfil its potential to affect clinical practice.

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'Genomics Research' generiert und verwendet enorme Datenmengen. Zur Analyse, aber auch zur Nutzung dieser Daten bei der Planung von genetischen Tests am einzelnen Patienten, ist der Forscher, wie auch der akademische Kliniker, auf Unterstützung durch Informationstechnologie (IT) angewiesen. Es hat sich das Fach der Bioinformatik etabliert. Die folgende Originalarbeit beschreibt eine solche Anwendung genetischer Forschung im Sinne von individualisierter Medizin durch Unterstützung von Bioinformatik. Es wird des weiteren aufgezeigt, wie durch rationale Anwendung bioinformatischer Techniken zahlreiche Experimente 'in-siliko' statt 'in-vitro' durchgeführt werden können.

2.3. Originalarbeit:

Data Mining Empowers the Generation of a Novel Class of Chromosome-specific DNA Probes [4]

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Abstract

Probes that allow accurate delineation of chromosome-specific DNA sequences in interphase or metaphase cell nuclei have become important clinical tools that deliver life-saving information about the gender or chromosomal make-up of a product of conception or the probability of an embryo to implant, as well as the definition of tumor-specific genetic signatures. Often such highly specific DNA probes are proprietary in nature and have been the result of extensive probe selection and optimization procedures. We describe a novel approach that eliminates costly and time consuming probe selection and testing by applying data mining and common bioinformatics tools. Similar to a rational drug design process in which drug-protein interactions are modeled in the computer, the rational probe design described here uses

a set of criteria and publicly available bioinformatics software to select the desired probe molecules from libraries comprised of hundreds of thousands of probe molecules. Examples describe the selection of DNA probes for the human X and Y chromosomes, both with unprecedented performance, but in a similar fashion, this approach can be applied to other chromosomes or species.

Keywords: X-linked diseases; Gender determination; Aneuploidy; Cytogenetics; Fluorescence in situ hybridization; Prenatal diagnosis; DNA probes; Rational probe design; Data mining; Genomics

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Introduction

Fluorescence in situ hybridization (FISH) is an established laboratory method to delineate specific nucleic acid sequences within interphase cell nuclei or metaphase spreads, which has proven indispensible for the detection of specific features in DNA and a variety of chromosomal rearrangements in cytogenetic and cancer research [1-3]. In the typical procedure, non-isotopically labeled nucleic acid probes are prepared first, and hybridized to denatured, single-stranded DNA or RNA targets, before unbound probe molecules are removed by wash steps. The bound probes can be seen in a fluorescence microscope equipped with filter combinations that match the excitation and emission properties of the fluorescent reporter molecules. Several targets can be tagged or 'decorated' simultaneously, and seen as individual hybridization signals, if the probes were labeled with different haptens [4-7].
The studies described below focus on the detection of DNA targets, and more specifically on detection, scoring or enumeration of specific chromosomes in interphase cell nuclei. This type of investigation finds clinical applications in a variety of fields. For example, if one of the parents of an unborn child, embryo or fetus is a known carrier of one of the many known recessive X-linked diseases, the chances of the offspring to be affected by the disease can be better prognosticated by determining its gender [8-10]. This is a relevant issue: according to Online Mendelian Inheritance in Man (OMIM), as of February 22, 2011, the current estimate of sequenced X-linked genes has reached 620 and the total including vaguely defined traits is an estimated 1138 [11].
Another common use of Y chromosome-specific DNA probes is the identification and tracking of grafted cells in sex-mismatched xenograft studies. For example, when Y chromosome bearing white blood cells from a male donor are transplanted to a female recipient, minute amounts of grafted cell can be identified following cell harvest and hybridization due to the presence of the Y-specific signal [12-15].

Materials and Methods

All procedures involving human subject have been reviewed and approved by the UC Berkeley Institutional Review Board.

Retrieval of target sequence for the Y probe

The nucleic acid sequence used for data mining was defined in our previous studies on *in vitro* DNA amplification of Y chromosome-specific DNA repeat sequences. Specifically, we designed pairs of oligonucleotide primers to amplify stretches of 124

bp from the 3.6 kb pentanucleotide DNA repeat described by Nakahori et al. (DYZ1, Genbank accession number X06228) [16,17] (Table 1). Primer annealing sites were chosen that had minimal homology with the human satellite III DNA repeat consensus sequence 'TTCCA' [16,18-21]. Blood samples from six normal human volunteers were used to validate the Y chromosome-specific PCR assay [22,23].
Serial cell dilution experiments and artificial mixing of flow-sorted Y chromosome carrying cells in predetermined aliquots of white blood cells from female donors determined that the primer combination WYR 4 (5'-GAACCGTACGATTCCATTCCTTTTGAA-3') – WYR 6 (5'-TTCCATTCCATTCCATTCCTTTCCTTT-3') amplifying a 248 bp DNA fragment corresponding to position 2965-3212 in Genbank accession number X06228 was sufficiently specific to detect a single male cell in the background of 1 million female cells [17,20,23]. Samples comprised entirely of female cells did not yield this product [20-23].

Database searches
We screened the National Center for Biotechnology Information (NCBI) human genome nucleotide DNA database for homologous sequences with one of the most widely used bioinformatics programs, Basic Local Alignment Search Tool (BLAST) [24]. The BLAST approach to rapid sequence comparison directly approximates alignments that optimize a measure of local similarity, the maximal segment pair (MSP) score. The basic algorithm is simple, robust and versatile; it can be implemented in a number of ways and applied in a variety of contexts including straightforward DNA and protein sequence database searches, motif searches, gene identification searches, and in the analysis of multiple regions of similarity in long DNA sequences [24,25].
Execution of the BLAST querying the human genome database with the 27-nucleotide (nt) sequence 'ATTCCGTACGATTCCATTCCTTTTGAA'.from position 3089-3115 of the human Y-specific 3564 bp repeat (Genbank accession number X06228) performed at http://ncbi.nih.gov/blast retrieved multiple hits. Parameters were set to identify clones with a range of levels of nucleic acid homology (setting: 'Optimize for somewhat similar sequences', (BLASTn)).

Retrieval of probe information for the pericentromeric region of the X chromosome
We used the University of California Santa Cruz (UCSC) Genome Browser GRCh37/hg19, built Februar 2009, at the University of California, Santa Cruz, accessible at http://genome.ucsc.edu/ to identify bacterial artificial chromosome (BAC) clones with high satellite DNA content [26]. The graphic user interface was set to display BAC end pairs and repeat DNA elements in the pericentromeric region of the human X chromosome, i.e., from position 58,232,531 bp to position 61,922,800 bp.

DNA probe preparation
The BAC DNAs were extracted from overnight cultures following an alkaline lysis protocol [27] or using a ZR BAC DNA Miniprep Kit (Zymo Research; Irvine, CA). The isolation of high molecular weight BAC DNAs was confirmed on 1% agarose gels and quantitated by Hoechst fluorometry using a Hoefer TK 100 instrument (Hoefer; South San Francisco, CA). Probe DNAs were labeled with biotin-14-dCTP (Invitrogen; Carlsbad, CA), digoxigenin-11-dUTP (Roche Molecular Systems; Indianapolis, IN), Spectrum Orange-dUTP (Abbott; Abbott Park, IL) or Spectrum Green-dUTP (Abbott) by random priming using a commercial kit (BioPrime Kit, Invitrogen)[7,28]. When incorporating fluorochrome-labeled deoxynucleoside triphosphates, the dTTP to

Spectrum Orange-dUTP or Spectrum Green-dUTP ratio in the reaction was adjusted to 2:1 [28-30].

Fluorescence *in situ* hybridization (FISH) and detection of bound probes

For in situ hybridization experiments, 1 µl of labeled DNA probe, 1 µl of salmon sperm DNA (10 mg/ml, 5 Prime – 3 Prime Inc.; Boulder, CO), 1 µl water, and 7 µl of the hybridization master mix (78.6% formamide (FA) (Invitrogen), 14.3% dextran sulfate (Sigma; St. Louis, MO) in 1.43x SSC, pH 7.0) (20x SSC is 3 M sodium chloride and 300 mM tri-sodium citrate, the pH was adjusted to 7.0) were combined, thoroughly mixed and denatured at 76°C for 10 min. The final concentration in the hybridization mixture is 55% FA, 10% dextran sulfate and 1xSSC, pH 7.0 [29, 30]. Acetic acid:methanol (1:3, vol.:vol.) fixed metaphase spreads were prepared on slides from phytohaemagglutinin-stimulated short-term cultures of peripheral blood lymphocytes from a karyotypically normal male following published procedures [31, 32]. Just prior to hybridization, slides were denatured for 4 min in 70% FA/2x SSC, pH 7.0 at 76°C, dehydrated in a 70%, 85%, 100% ethanol series, two minutes each step, and air dried. About 2.5 µl of hybridization mixture were placed on the denatured samples, covered with a 12 mm diameter circular coverslip, sealed with rubber cement and incubated for 16-20 hours at 37°C in a humidified chamber.

After the rubber cement was carefully removed from the slides, the coverslip was allowed to slip off in 2x SSC at 20°C. Post-hybridization washes were performed as described [7,28] and included two washes in 50% FA/2x SSC buffer at 43°C for 15 min each followed by two washes in 2x SSC at 20°C [33,34]. If non-fluorescently-labeled DNA probes were hybridized, i.e., the biotinylated or digoxigenin-labeled probes, unspecific binding sites were blocked with PNM buffer (5% nonfat dry milk, 0.1% NaN_3 in PN buffer (0.1M sodium phosphate buffer, pH 8.0, plus 0.05% NP-40))[7,28] for 10 min, before probes were detected with either fluorescein-conjugated avidin DCS (Vector; Burlingame, CA) or anti-digoxigenin rhodamine (Roche). An additonal two washes in 2x SSC were applied to remove unbounded avidin or antibodies. Finally, the slides were mounted with 4,6-diamino-2-phenylindole (DAPI) (0.1µg/ml) in antifade solution (0.1% p-phenylenediamine dihydrochloride (Sigma), 0.1x phosphate buffered saline (Invitrogen), 45 mM $NaHCO_3$, 82% glycerol (Sigma), pH 8.0) and coverslipped.

Image acquisition and analysis

Fluorescence microscopy was performed on a Zeiss Axioskop microscope (Zeiss, New York, NY) equipped with a filter sets for observation of Cy5/Cy5.5, Texas red/rhodamine, FITC or DAPI (84000v2 Quad, ChromaTechnology; Brattleboro, VT). Images were collected using a CCD camera (VHS Vosskuehler; Osnabrueck, Germany) and processed using Adobe Photoshop® software (Adobe Inc.; Mountain View, CA).

Results and Discussion

Chromosome Y BAC clone selection and validation

The bacterial artificial chromosome clone with the highest homology score, RP11-242E13 (Genbank accession number AC068123) contains 28 identical copies of 23 of the 27 nt query sequence in its 98295 bp insert (Fig.1).

```
gb|AC068123.5|AC068123 Homo sapiens BAC clone RP11-242E13 from Y, complete sequence
Length=98295
Score = 46.1 bits (23), Expect = 0.003
Identities = 23/23 (100%), Gaps = 0/23 (0%)
Strand=Plus/Plus
Query 1 ATTCCGTACGATTCCATTCCTTT 23
|||||||||||||||||||||||
Sbjct 3112 ATTCCGTACGATTCCATTCCTTT 3134
Score = 46.1 bits (23), Expect = 0.003
Identities = 23/23 (100%), Gaps = 0/23 (0%)
Strand=Plus/Plus
Query 1 ATTCCGTACGATTCCATTCCTTT 23
|||||||||||||||||||||||
Sbjct 6670 ATTCCGTACGATTCCATTCCTTT 6692
Score = 46.1 bits (23), Expect = 0.003
Identities = 23/23 (100%), Gaps = 0/23 (0%)
Strand=Plus/Plus
Query 1 ATTCCGTACGATTCCATTCCTTT 23
|||||||||||||||||||||||
Sbjct 10234 ATTCCGTACGATTCCATTCCTTT 10256
Score = 46.1 bits (23), Expect = 0.003
Identities = 23/23 (100%), Gaps = 0/23 (0%)
Strand=Plus/Plus
Query 1 ATTCCGTACGATTCCATTCCTTT 23
|||||||||||||||||||||||
Sbjct 13823 ATTCCGTACGATTCCATTCCTTT 13845
Score = 46.1 bits (23), Expect = 0.003
Identities = 23/23 (100%), Gaps = 0/23 (0%)
Strand=Plus/Plus
Query 1 ATTCCGTACGATTCCATTCCTTT 23
|||||||||||||||||||||||
Sbjct 17357 ATTCCGTACGATTCCATTCCTTT 17379
Score = 46.1 bits (23), Expect = 0.003
Identities = 23/23 (100%), Gaps = 0/23 (0%)
Strand=Plus/Plus
Query 1 ATTCCGTACGATTCCATTCCTTT 23
|||||||||||||||||||||||
Sbjct 20831 ATTCCGTACGATTCCATTCCTTT 20853
Score = 46.1 bits (23), Expect = 0.003
Identities = 23/23 (100%), Gaps = 0/23 (0%)
Strand=Plus/Plus
Query 1 ATTCCGTACGATTCCATTCCTTT 23
|||||||||||||||||||||||
        Sbjct 24410 ATTCCGTACGATTCCATTCCTTT 24432
```

Figure 1: The results of a BLASTn query using a human Y chromosome-specific 27nt DNA sequence. Shown are the first seven hits of the alignment search, which in total resulted in 28 perfect matches for 23/27 nt query sequence.

According to the NCBI database, this clone has been mapped to the long arm of the Y chromosome, band q12 (Table 1).

Table 1. BAC clones for human chromosomes X and Y

Clone numbers	Band	Start (bp)	End (bp)	Insert size (bp)	Database	Accession
RP11-242E13	Yq12	58819362	58917656	98295	NCBI	AC068123
RP11-294C12	Xq11.1	61696702	61722561	25860	UCSC	AQ508215, AQ508213
RP11-348G24	Xp11.1	58356061	58564667	208607	UCSC	AQ528470, AQ528473

With one exception (hit #27), the 23 bp sites (Fig.1) are spaced in more-or-less regular intervals of slightly more than 3500 bp (Table 2).

Table 2. Location of 28 BLASTn hits with the 27 nt query sequence in BAC RP11-242E13

Hit	Position in RP11-242E13	Distance to previous hit (bp)
1	3112	Not applicable.
2	6670	3558
3	10234	3564
4	13823	3589
5	17357	3534
6	20831	3474
7	24410	3579
8	27985	3575
9	31579	3594
10	35143	3564
11	38702	3559
12	42276	3574
13	45858	3582
14	49452	3594
15	53026	3574
16	56570	3544
17	60159	3589
18	63713	3554
19	67242	3529
20	70826	3584
21	74400	3574
22	77984	3584
23	81543	3559
24	85107	3564
25	88680	3573
26	92264	3584
27	94258	1994
28	97817	3559

The site #27 is spaced 1994 bp from the previous site of homology with the query sequence, thus indicating a single incomplete 3.5kb DNA repeat in BAC RP11-242E13. Without including site #27, the sites are spaced an average of 3566 bp, which is in excellent agreement with the published sequence for a single repeat of 3564 bp described by Nakahori et al. [16].

Validation of the Y-specific BAC clone RP11-242E13 by FISH

The result of an in situ hybridization experiment using a biotinylated DNA probe prepared from BAC clone RP11-242E13 is shown in Figure 2A. As anticipated, the 98 kb DNA probe decorated the distal long arm of the human Y chromosome suggesting that there are hundreds or even thousands of copies of the complementary DNA target sequence [35] (Figure 2A).

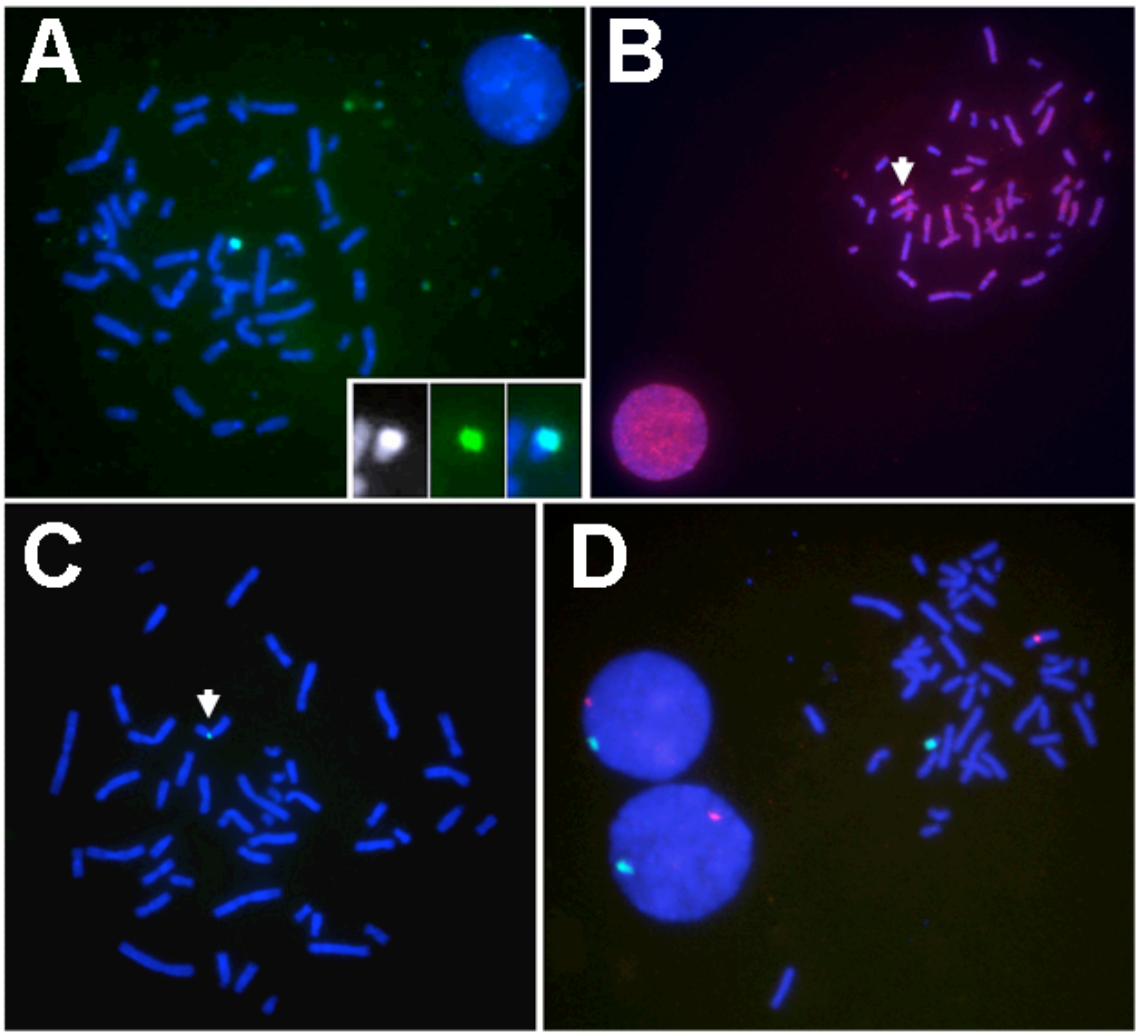

Figure 2: Clone validation by FISH on to normal male human metaphase spreads and interphase cells. (A) In situ hybridization of a biotinylated probe prepared from BAC RP11-242E13 exhibited strong signals on the long arm the human Y chromosome (arrow). The insert shows an enlarged picture of the Y chromosome with the DAPI picture to the left, the FITC picture in the center and a superposition of both on the right. **(B)** A digoxigenin-labeled DNA probe prepared from BAC RP11-348G24

resulted in specific signals on the X chromosome (arrow) in the presence of a significant amount of cross-hybridization to other chromosomes. Please note the high level of cross-hybridization in the interphase nucleus shown in the lower right corner. **(C)** In contrast to the result shown in (B), a probe prepared from BAC RP11-294C12 bound virtually exclusively to the centromeric heterochromatin of the human X chromosome (arrow). **(D)** Multiplex analysis of human gonosomes was achieved by combining differently labeled DNA probes in a single hybridization experiment. In this experiment, the Y chromosomal target appears in green (arrow), while the X-chromosomal centromeric repeat DNA is shown decorated in red (arrow).

Interestingly, even in this simple hybridization experiment that did not include any type of blocking DNA, the probe lit up just the chromosome Y, band q12. No cross-hybridization with other chromosomes that are known to carry satellite III-type heterochromatin such as human chromosome 9 [19] was found. In summary, our findings suggest that BAC clone RP11-242E13 carries multiple copies of the 3.6kb DNA repeat which hybridize exclusively to the human Y chromosome.

BAC clones from the pericentromeric region of the X chromosome

The data mining effort searching for a large insert, recombinant DNA clone that hybridizes specifically to the heterochromatic DNA tandem repeats in the peri-centromeric region of the human X chromosome was guided by our prior observation that chromosome-specific alpha satellite DNA repeats can be identified in BAC clones through database searches [36]. We had noticed that alpha satellite DNA repeats which typically appear as chromosome-specific, high order tandem repeats in the peri-centromeric region of human autosomes [26, 37] can also be found dispersed in low copy numbers in some significant distance from the centromeric regions and in vitro DNA amplification can be used to enrich the alpha satellite sequences [36].
The graphical user interface of the UC Santa Cruz Genome Browser suggested that several clones from the RP11 BAC library map into the X chromosome-specific pericentromere and contain alpha satellite DNA repeats (Figure 3).

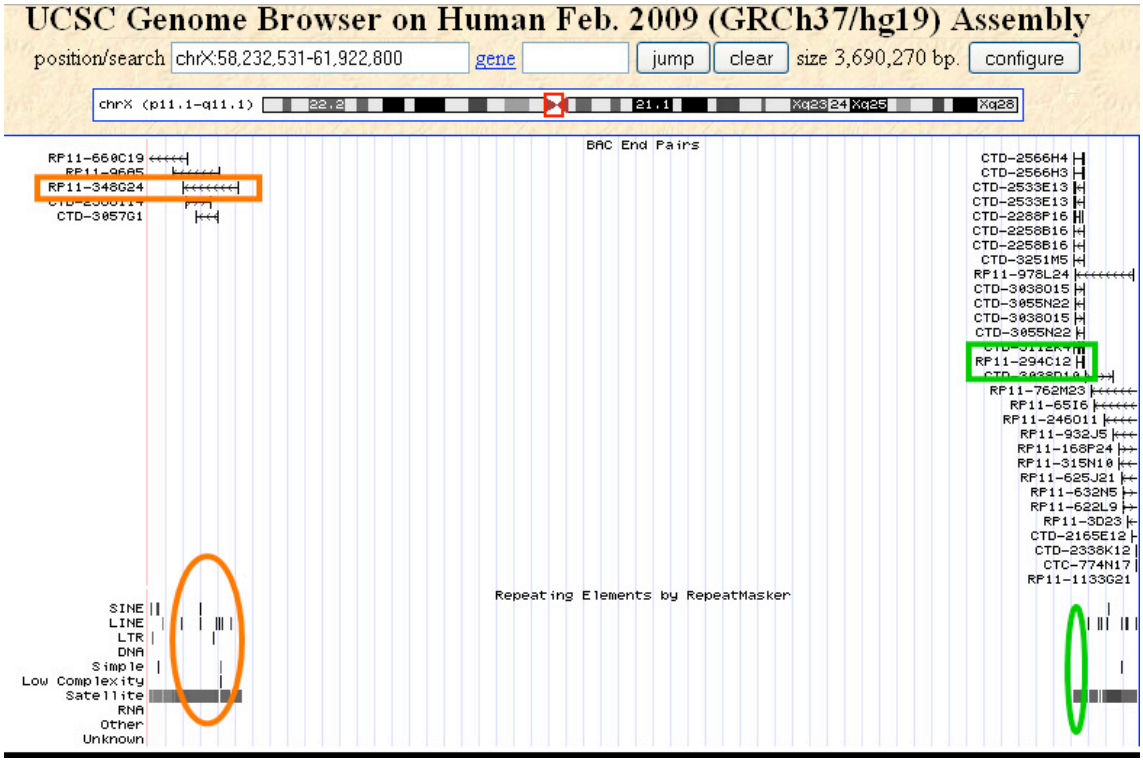

Figure 3: Selection of an X chromosome-specific BAC clone using the UCSC Genome Browser.

On top, the figure depicts a X chromosome ideogram with the selected pericentromeric region 58,232,531-61,922,800 bp highlighted by a red square. The lower part of the picture shows BAC end alignments within this interval as well as the locations of DNA repeat sequences (bottom). Clones and DNA repeat sequences of interest are indicated by colored squares and circles, respectively. Please note the extensive area of central, unchartered DNA representing the centromere.

We decided to test two BAC clones, one that is essentially free of interspersed DNA repeats such as short or long interspersed element (LINE) sequences (RP11-294C12) mapping to chromosome X, band q11.2 (Table1) and a second clone (RP11-348G24), which contains a few interspersed DNA repeats and maps to chromosome X, band p11.1 (Figure 3). After retrieval from the freezer, both clones grew in Luria Bertoni broth at approximately the same rate. The DNA yields from 5 ml overnight cultures were similar, but the labeled DNAs showed drastically different hybridization patterns. The results are shown in Figures 2B and 2C.

The digoxigenin-labeled probe prepared from BAC clone RP11-348G24 containing the interspersed repeats gave a strong signal on the X chromosome, but also unacceptably high levels of cross-hybridization with other autosomes (Figure 2B). Many interphase nuclei exibited such high levels of cross-hybridization that it was not possible to delineate the X chromosome target. In contrast, hybridization of a biotinylated DNA probe prepared from BAC clone RP11-294C12 resulted in signals that localized exclusively to the pericentromeric region of the human X chromosome (Figure 2C).

To demonstrate that the highly specific DNA probes for chromosomes X and Y can be combined in dual-color multiplex hybridization experiments [38-40], we labeled the Y chromosome-specific DNA from BAC clone RP11-242E13 with Spectrum Green-dUTP (green fluorescence) and the X chromosome-specific DNA from BAC clone RP11-294C12 with Spectrum Orange-dUTP (red fluorescence). Hybridization of this probe mixture gave strong, specific signals in metaphase as well as interphase cells (Figure 2D).

Probe generation and testing rates
Turnaround times for generation and validation of DNA probes prepared from BAC's are at least one order of magnitude shorter than those observed with yeast artificial chromosome clones as large insert human genomic DNA probe template [41-43].

Conclusions

This study was undertaken to specifically seek answers to three questions:

1. Can researchers with limited access to computational capabilities data mine and take advantage of the huge resources generated by the International Human Genome Project?
2. How can one identify recombinant, large insert DNA clones with features that make them ideal hybridization probes? and
3. What constitutes an inexpensive and rapid approach to validate the selected clones?

We believe that all three questions have been answered in this study. First, we have been able to demonstrate that simple, publicly available bioinformatics tools such as BLAST searches running on a simple desktop computer allow the operator to data mine and extract the desired information from publicly available archives. Secondly, we began to define rules to predict DNA probe properties based on DNA sequence analysis. Lastly, we were able to show that in a matter of days, chromosome-specific DNA probes can be defined, prepared and validated by FISH.

The importance of probe specificity can not be overemphasized. While recombinant DNA clones carrying large chunks of the human genome such as the BAC or P1 clones (39, 40,43-44) are easy to propagate, the presence of non-chromosome-specific interspersed DNA repeats can lead to major impediments in FISH-based interphase cell analysis [36]. As shown above, careful probe selection based on mining of the publicly accessible databases may circumvent some of these problems.

The simple, yet efficient data mining approach presented in this paper is our first step towards creation of an 'in silico' DNA probe set that may find extensive use in the analysis of NexGen, deep sequencing data [45,46]. Thus, this new class of 'in silico probes' has little or nothing in common with the conventional recombinant DNA probes [1,2,4,19,47], many of which have not even been sequenced, and it is more akin to the well known 'in silico PCR' [48]. Here, we describe a software-based interactive approach in which the user checks the results of the data mining operation and validates the BAC clones via FISH. In our lab setting, the approach allowed us to select and validate a few clones (<10) per week.

In closing, it's important to mention that polymorphisms or centromeric heteromorphisms might present serious problems in the FISH-based analysis of cells from affected individuals using DNA repeat probes [49] regardless whether such an analysis is performed by convention FISH, Spectral Imaging or multicolor FISH [6,34,50]. The before-mentioned deep sequencing in combination with 'in silico' copy number analysis might prove to be a reasonable alternative approach.

Acknowledgements
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Disclaimer

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Eine Aneusomie, also eine abnorme Anzahl von Chromosomen, ist ein Risikofaktor für pathologisches Zellverhalten. In einer konsequenten Weiterentwicklung der zuvor beschriebenen bioinformatischenTechniken, stellt die folgende Arbeit eine praktische klinische Applikation des Algorhythmus vor.

2.4. Originalarbeit:

Bioinformatic Tools Identify Chromosome-Specific DNA Probes and Facilitate Risk Assessment by Detecting Aneusomies in Extra-embryonic Tissues [5]

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Running Title: Placenta Probe Identification and Validation

Abstract

Despite their non-diseased nature, healthy human tissues may show a surprisingly large fraction of aneusomic or aneuploid cells. We have shown previously that hybridization of three to six non-isotopically labeled, chromosome-specific DNA probes reveals different proportions of aneuploid cells in individual compartments of the human placenta and the uterine wall. Using fluorescence in situ hybridization, we found that human invasive cytotrophoblasts isolated from anchoring villi or the uterine wall had gained individual chromosomes. Chromosome losses in placental or uterine tissues, on the other hand, were detected infrequently. A more thorough numerical analysis of all possible aneusomies occurring in these tissues and the investigation of their spatial as well as temporal distribution would further our understanding of the underlying biology, but it is hampered by the high cost of and limited access to DNA probes. Furthermore, multiplexing assays are difficult to set up with commercially available probes due to limited choices of probe labels. Many laboratories therefore attempt to develop their own DNA probe sets, often duplicating cloning and screening efforts underway elsewhere. In this review, we discuss the conventional approaches to the preparation of chromosome-specific DNA probes followed by a description of our approach using state-of-the-art bioinformatics and molecular biology tools for probe identification and

manufacture. Novel probes that target gonosomes as well as two autosomes are presented as examples of rapid and inexpensive preparation of highly specific DNA probes for applications in placenta research and perinatal diagnostics.

Key Words: Aneusomy, gestation, cytotrophoblast, fetal-maternal interface, bioinformatics, DNA probes, bacterial artificial chromosomes, fluorescence in situ hybridization (FISH).

Introduction

The human placenta is a vital organ anchoring the fetus to the mother via the uterus and providing an interface for the transport of nutrients, gases and waste. The overwhelming number of chromosomal studies of the placenta has been performed on cells biopsied from floating villi, which were cultured for several days to obtain metaphase spreads for conventional chromosome banding analysis. We decided to perform investigations on uncultured interphase cells using fluorescence in situ hybridization (FISH), since cell viability or proliferation are minor concerns when using FISH [1-5]. Probes for our initial studies of aneuploidy in extra-embryonic tissues were obtained from a commercial source (Abbott, Des Moines, IL)[6, 7]. Probe sets were comprised of three to four chromosome enumerator probes (CEPs) targeting chromosome types, X, Y, 16 or 18, or locus-specific probes (LSPs) for chromosome 13 or 21 [7]. Studying the chromosomal make-up of cells in different compartments of anchoring villi and the uterine wall also referred to as 'basal plate', we found that the karyotypes of these extra-embryonic cells were mostly unrelated to the karyotype of the fetus [5, 7, 8]. The most common abnormality we have observed was a gestational age-related gain of chromosomes affecting invading cytotrophoblasts (iCTB's)[7]. For a more comprehensive analysis and to be able to increase the number of chromosome types that can be scored simultaneously in a single FISH experiment, we had to develop our own custom sets of chromosome-specific DNA probes.
While the DNA probe development efforts described in the present communication were prompted by the need to develop a novel probe set for more comprehensive cytogenetic analyses of normal placental tissue compartments from uncomplicated pregnancies [6], DNA probes selected in a similar fashion are likely to find widespread application in investigations of unusual conditions such as spontaneous abortions [9, 10] or confined placental mosaicism (CPM) [11-14], the cytogenetic analysis of human preimplantation embryos [15-21], perinatal analysis [22-24], tumor research and diagnosis [1-5, 25-27] as well as radiobiological or environmental studies [28-40]. Thus, the description of our probe selection approach combining bioinformatics tools for data mining of genomic databases with deeply redundant recombinant DNA clone libraries, which follows the brief review of the more conventional techniques for DNA probe selection, may provide useful information for a diverse group of researchers in the life sciences and enable the average research lab to prepare chromosome-specific custom DNA probes at a very affordable cost.

Selection of DNA Target Sequences and Preparation of non-isotopically Labeled DNA Probes for FISH

Briefly, successful cytogenetic analysis by FISH is based on the formation of stable hybrids between the DNA targets inside cell nuclei or metaphase chromosomes and the

labeled DNA probes molecules provided by the investigator [41]. The DNA probes can either be marked by a fluorochrome, which can then be detected by eye or a camera attached to a fluorescence microscope, or by a non-fluorescent, non-isotopical hapten, most often biotin, digoxigenin or dinitrophenol, which is detected by a fluorescent moiety such as a fluorochrome-labeled avidin or antibody. Different probe types are available to suit particular applications: whole chromosome painting probes allow the delineation of inter-chromosomal translocations in metaphase spreads [37, 42, 43], while intra-chromosomal rearrangements are detected in metaphase or interphase cells with chromosome band-specific probes [44-47]. In addition, there are DNA probes that target somewhat smaller, gene- or locus-specific regions [34, 48-52].
While the FISH technology found widespread application in research laboratories around the world, its acceptance in clinical settings is still hampered by a limited selection of commercially available, U.S. Food and Drug Administration (FDA)-approved tests and the typically labor-intensive, costly nature of producing DNA probes that perform well in multiplexed assays [53]. While FDA approval may be required for all diagnostic probes that are shipped across state borders in the U.S., the in-house preparation of DNA probes might lead to significant cost savings in research laboratories. Our laboratories have a long-standing track record of production of novel DNA probes and innovative cytogenetic assays, many of which have found their way into contemporary cancer research or PGD analysis [16, 43, 45, 47, 48, 50, 54-60]. To facilitate the distribution of molecular cytogenetic assays and make DNA probes as well as multiplex FISH tests available to the less experienced laboratory, we have undertaken probe production pilot studies, which take advantage of the vast resources generated in the course of the Human Genome Project such as physical maps and recombinant DNA libraries.

Our initial studies focused on the preparation of novel DNA probes for chromosome scoring or ‘enumeration’ in interphase cell nuclei and metaphase spreads, since these seem to remain the most common applications in research and the clinical settings [53, 61]. The vast majority of these CEPs target highly reiterated, tandemly-repeated DNA sequences in order to bind many copies of a rather small probe sequence to a tightly localized area or volume. Different ways of isolating and purifying such DNA probes exist [25, 54, 59, 60, 62-66].
Briefly, up until the 1980’ies, satellite DNA sequences were enriched, isolated and characterized by a cumbersome, labor-intensive workflow which involved either density gradient centrifugation or timed reassociation of single stranded, thermally denatured DNA followed by enzymatic digestion of single stranded DNA by exonucleases. This was followed by molecular cloning, library screening, clone characterization and DNA sequencing which made this a rather costly enterprise [67-69]. The use of endonucleases to break up large tandemly repeated DNA clusters facilitated the hunt for chromosome-specific heterochromatic, satellite DNA, expedited the cloning-characterization steps and lead to major progress in the identification of chromosome-specific high order tandem repeats [62, 70-74].
The breakthrough in the isolation of chromosome-specific DNA polynucleotides and preparation of DNA probes for FISH came with the application of DNA amplification using the polymerase chain reaction (PCR) in the late 1980’ies: chromosome-specific sequences could be extracted on-line from larger, high order tandem repeats of satellite DNA to define the PCR primer sequences and amplify a specific fragment from genomic DNA [54](Fig.1A).

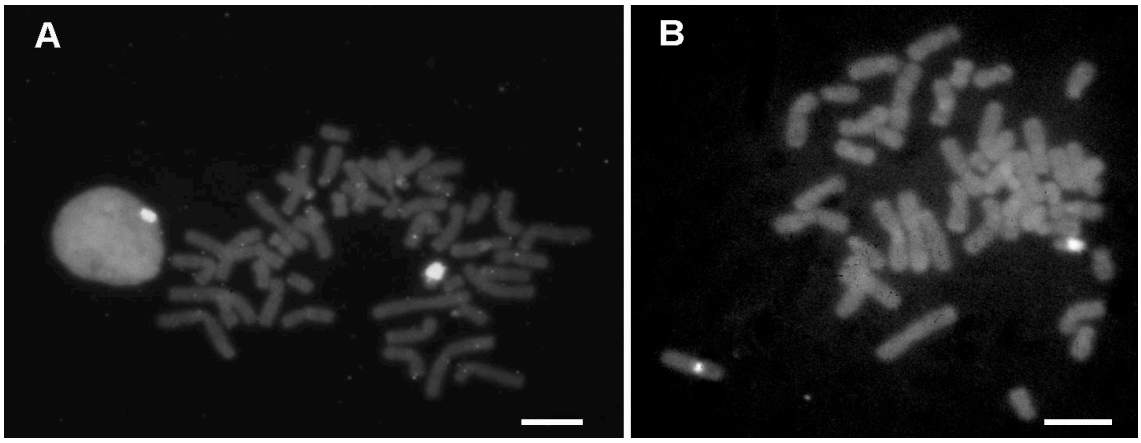

Fig. (1). In situ hybridization of cloned, chromosome-specific PCR products.
A) Biotinylated DNA prepared from PCR products with chromosome Y -specific oligonucleotide primers bind specifically to the heterochromatic region of the human Y chromosome.
B) The Y-specific probe shown in A) can be combined with a biotinylated probe for the smaller tandemly repeated DNA cluster at the centromeric region of the X-chromosome. Bound probes were detected with avidin-FITC (green fluorescence) on ethidium bromide (red fluorescence) stained chromosomes, here shown as grey scale images. (Bars = 10 mm.)

In a variation of this scheme, chromosome-specific sequences could be amplified with consensus PCR primers from template DNA which provided limited sequence variety, such as flow-sorted human or mouse chromosomes [25, 75](Fig.1B). In general, DNA probes generated this way still represented a pool of diverse sequences and molecular cloning was required to isolate the highly specific, informative probes [25].

It wasn't until the completion of a first draft of the human genome sequence when new sets of genomic tools became available that would revolutionize the ways individual investigators analyze the human genome in the 1990'ies and onwards often using no more than their personal computer and an on-line connection to publicly available databases. Large insert, recombinant DNA libraries such as YAC [76, 77], P1 [78, 79]or BAC [66, 80, 81] libraries had been constructed and characterized, clones had been end-sequenced and placed on the larger physical maps by basic sequence alignment procedures [82].

The work of Baumgartner et al. (2006) [65] showed that a combination of database searches (to identify BAC clones rich in satellite content) in combination with in vitro DNA amplification can expedite the preparation of chromosome-specific DNA probes. However, this approach still requires some *a priori* knowledge of the target sequence to specify the PCR primers [65].
We recently demonstrated that publicly available on-line databases can be analyzed using a suite of simple bioinformatics tools to identify chromosome-specific BAC clones [60]. Specifically, we used our proprietary information of a Y chromosome-specific sequence [83-85] and a DNA sequence alignment program (BLAST) [82] to identify BAC clone RP11-243E13 as a potential DNA probe. Using the Genome Browser program at the UC Santa Cruz (UCSC) Genome Center web site

(genome.ucsc.edu), we then identified a BAC clone mapped to the satellite containing centromeric heterochromatin on the human X chromosome (BAC RP11-348G24) [60]. Probes prepared from these two BAC clones showed an impressive better-than-expected performance in FISH experiments by displaying strong, highly specific FISH signals localized exclusively to the target chromosomes (Fig.2).

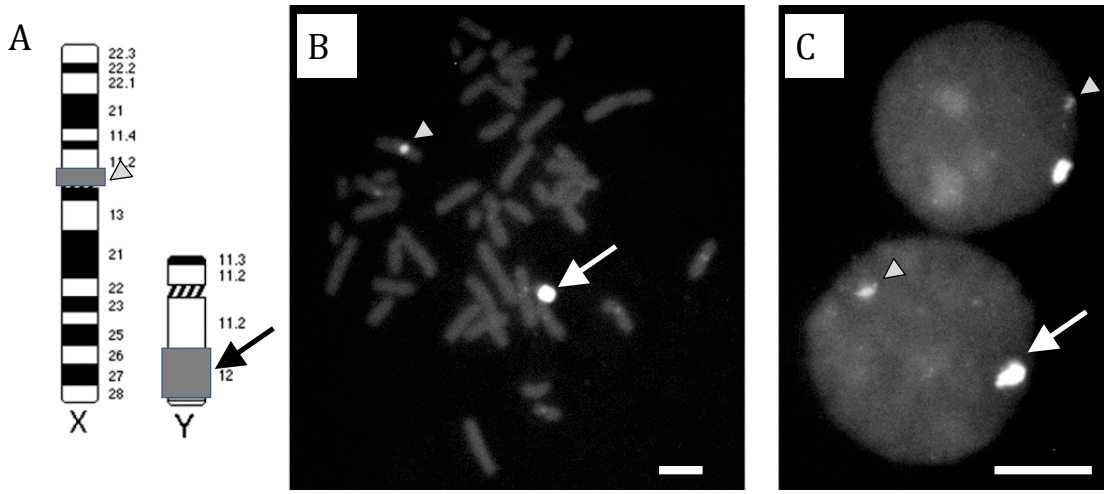

Fig. (2). In situ hybridization analysis of DNA probes prepared from BAC clones. The BAC clones RP11-348G24 and RP11-242E13 hybridized to metaphase spreads prepared from short term cultures of human lymphocytes showed specific hybridization to the target regions on the X (arrowhead) and Y (arrow) chromosome, respectively. **A)** Schematic representation of the FISH target regions on the X and Y chromosome. **B)** Hybridization of both probes to metaphase chromosomes. **C)** Hybridization signals in diploid interphase cell nuclei. (Bars = 10 mm.)

Probe Preparation and Fluorescence in situ Hybridization (FISH) of BAC-derived DNA Probes

The procedures used for hybridization of BAC-derived DNA probes follow pretty much the published procedures for oligonucleotide, plasmid or P1-derived DNA probes [50, 86, 87]. In typical experiments, the BAC DNAs are extracted from overnight cultures following an alkaline lysis protocol [88] or using a BAC DNA miniprep kit (Zymo Research; Irvine, CA). The DNAs are confirmed on a 1% agarose gel and quantitated. Probe DNAs are labeled with biotin-14-dCTP or digoxigenin-11-dUTP (Roche; Indianapolis, IN) by random priming using a commercial kit (BioPrime Kit, Invitrogen; Carlsbad, CA). Slides of metaphase spreads of cells are made from short-term cultures of peripheral blood lymphocytes from a karyotypically normal male following published procedures [35].

The slides (metaphase cells, interphase cell nuclei or slides carrying deparaffinized tissue section) are denatured in 70% formamide at 70 °C, dehydrated and overlaid with a hybridization cocktail containing 20-50 ng of denatured probe DNA in buffer containing 10% dextran sulfate and 50-55 % formamide. Following overnight incubation at 37°C (48 or more hours for deparaffinized tissue sections), slides are washed to removed excess probes and incubated with a fluorochrome-conjugated avidin or corresponding antibodies as required [59, 66, 89]. Finally, the slides are mounted with 4,6-diamino-2-phenylindole (DAPI) (0.1µg/ml) in antifade solution coverslipped and imaged on the fluorescence microscope.

BAC-derived DNA Repeat Probes for Autosomal Targets

We were also interested in whether this concept of knowledge-based probe selection can be extended to probes for human autosomes. In our 2006 paper [65], we had proposed a satellite-rich BAC clone, RP11-469P16, as template for a PCR based probe generation scheme. The UCSC Human Genome Browser at genome.ucsc.edu indicates the presence of a long interspersed DNA repeat (LINE) in the BAC insert, which may lead to undesirable cross-hybridization since LINE's are not chromosome-specific, but exist in thousands of copies across the human genome.

According to information provided on the UCSC Genome browser web site, a BAC insert typically consists of 25-350 kb of DNA. During the early phase of a sequencing project, it is common to sequence a single read (approximately 500 bases) at each end of each BAC from a large library. Later on in the project, these BAC end reads are mapped *in silico* to the genome draft sequence. Tracks in the genome browser as shown in Fig.3 show these mappings in cases where both paired ends could be mapped within.

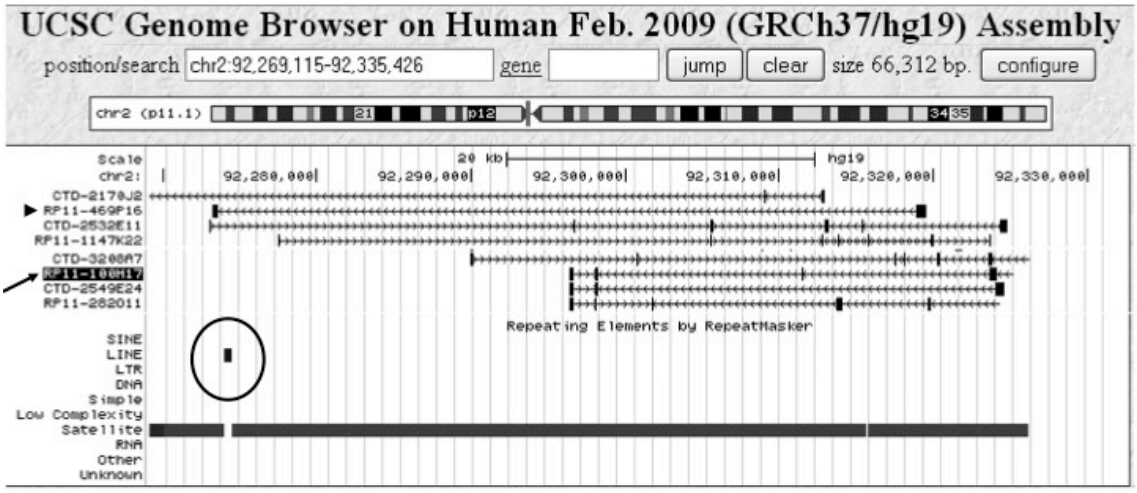

Fig. (3). Screen dump of the UCSC Genome Browser (GoldenPath) display showing the BAC ends mapped to the targeted region of chromosome 2: 92,269,115 bp to 92,335,426 bp. The alignment BAC clone end sequences with the draft sequence of the human genome places BAC RP11-100H17 (arrow) in a region comprised entirely of satellite DNA, while BAC RP11-469P16 (arrowhead) is predicted to contain a cluster of long interspersed DNA repeats (LINE's)(circled).

A valid pair of BAC end sequences must be at least 25 kb but no more than 350 kb away from each other. The orientation of the first BAC end sequence must be "+" and the orientation of the second BAC end sequence must be "-". BAC end sequences are placed on the assembled sequence using Jim Kent's blat program [90]. Tracks can be used for determining which BAC contains a given gene or DNA repeat clusters using the 'RepeatMasker' program (www.repeatmasker.org). Please note that for the heterochromatic regions, there

has been almost no clone validation in place to ensure that the BAC probe is the correct or predicted size or chromosomal locations.

When using a DNA probe prepared from BAC RP11-469P16, FISH results showed cross-hybridization to multiple chromosomes other than chromosome 2 (Fig.4A, B).

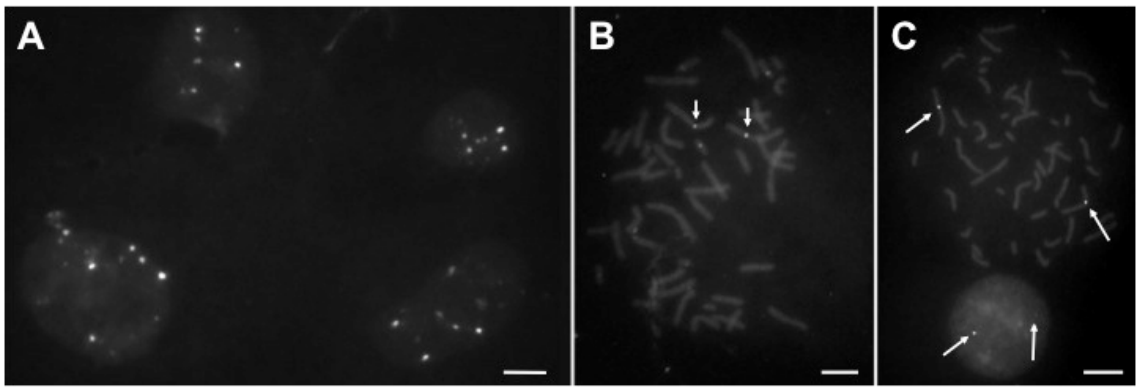

Fig. (4). FISH performance of BAC-derived DNA probes targeting the centromeric heterochromatin of chromosome 2. A-B) A DNA probe prepared from BAC clone RP11-469P16 shows multiple signals in normal interphase cell nuclei (A) or on metaphase spreads (B). Arrows in B) point at the target region on chromosome 2. **C)** A DNA probe prepared from BAC clone RP11-100H17 binds exclusively to the chromosome 2-specific target region (arrows). (Bars = 10 mm.)

However, a DNA probe prepared from BAC clone RP11-100H17 (Fig.3, arrow), which is expected to bind ~20 kb proximal of RP11-469P16 on the short arm of human chromosome 2, gave strong, highly specific FISH signals on interphase and metaphase cells (Fig.4C). This can be attributed to the lack of interspersed non-chromosome specific DNA repeats in the insert of BAC RP11-100H17 as well as it's composition of DNA tandem repeat units of entirely chromosome 2-specific satellite DNA.

Since inserts of BAC clones that contain satellite DNA, but no short or long interspersed DNA repeats (SINE's, LINE's) appear to render a high signal-to-noise ratio and strong chromosome specific signals which can easily be scored by eye using a microscope, we prepared a SINE-/LINE-free DNA probe for the short arm of chromosome 4, band p11. The BAC RP11-360M1 carries an insert of an estimated 59846 bp, which is rich in tandemly-repeated satellite DNA repeats, but free of interspersed repeat DNA (Fig. 5).

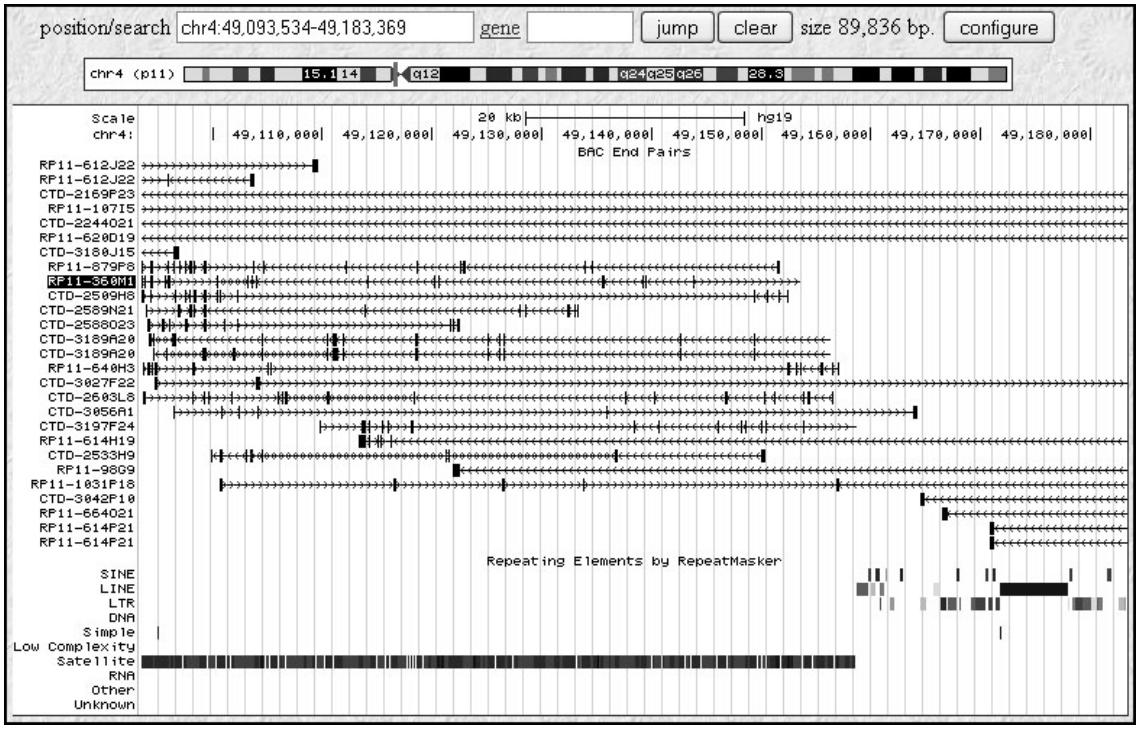

Fig. (5). Screen dump of the UCSC Genome Browser display showing the BAC ends mapped to the region chromosome 4: 49,093,534 bp to 49,183,369 bp. The alignment BAC clone end sequences with the draft sequence of the human genome places BAC RP11-360M1 (highlighted) in a region comprised almost entirely of satellite DNA and completely free of interspersed DNA repeats such as short interspersed repeats (SINE's) or LINE's.

In situ hybridization of the chromosome 4-specific DNA probe prepared from BAC RP11-360M1 in combination with a differently labeled probe for the centromeric region of the X chromosome BAC RP11-294C12 to deparaffinized human placental tissue section showed excellent probe performance, i.e., strong and highly specific DNA signals with were easily scored (Fig.6).

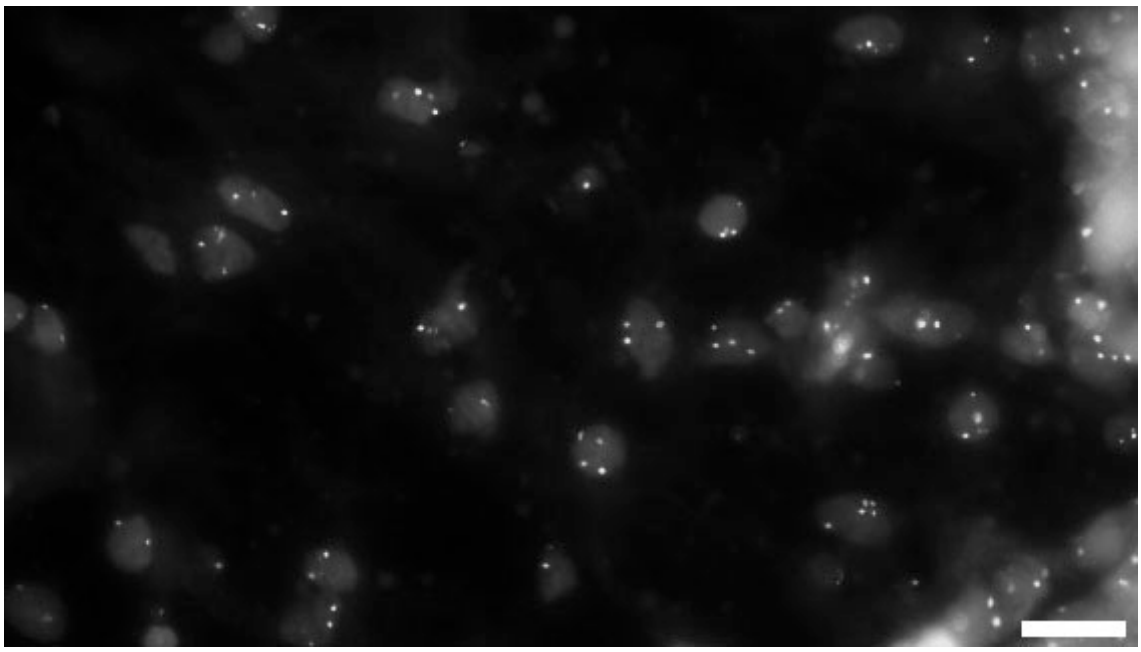

Fig. 6. FISH analysis of placental tissue sections. Probes prepared from BAC clones RP11-294C12 (confirmed target is chromosome X) and RP11-360M1 (supposed to bind to chromosome 4) give compact, easy-to-score hybridization signals on formalin-fixed, paraffin-embedded tissue sections. The red or green signals from the original image together with the DAPI counterstain are overlayed in this grayscale image. (Bars = 10 mm.)

Concluding Remarks

Molecular cytogenetic analyses using FISH have provided major contributions to our understanding of disease processes including tumorigenesis, cancer progression and metastasis, but also to the existence of aneuploid cell populations or cohorts in seemingly normal tissues [5, 61, 91-96].
For example, with an incidence of one in every 5-6 clinically recognized pregnancies, spontaneous abortions (SABs) during the first trimester are the most frequent pregnancy complication in women [9]. Causes of SABs have been identified as chromosomal abnormalities, uterine defects, immunological problems, hormonal imbalance and infections [2-6]. While more than half of all first trimester SABs are associated with chromosomal abnormalities, nearly 40% remain unexplained [6]. With no apparent association between placental villous morphology and fetal chromosomal abnormalities, SABs with either euploid or aneuploid conceptuses demonstrated incomplete cytotrophoblast (CTB) differentiation and compromised invasion [7-9]. These observations prompted our studies of the chromosomal make-up of extra-embryonic cells at materno-embryonic and fetal-maternal interfaces, i.e., the human placenta and the uterine wall. However, as mentioned in the introduction the application of DNA probes described in this review is not limited to investigations of fetal or extra-embryonic tissues.

The novel database mining approach to DNA probe selection described here is a fast and inexpensive solution to the problems of ‘probe bottlenecks’ in clinical research.

Mapping information for BAC clones is publicly available from UCSC or the National Center for Biomedical Information (NCBI)/National Institute of Health, USA, different libraries outside the US, such as the Wellcome Trust Sanger Institute, Hinxton, UK, or the Resources for Molecular Cytogenetics, Dipartimento di Genetica e Microbiologia, Universita' di Bari, Bari, Italy, as well as several commercial sources are available to purchase these clones. The BAC-derived satellite DNA probes also seem to out perform most of the chromosome enumerator probes that are presently in use in research and clinical laboratories. In summary, the procedures described in the present communication allow a laboratory with typical, non-specialist equipment to prepare chromosome-specific DNA probes in just a few days and thus represent the most efficient, rapid and cost-conscious approach to generation of chromosome-specific DNA probes for cytogenetic studies.

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Meine hier aufgeführten Forschungsergebnisse sind dem Bereich der 'Basic Science', also der Grundlagenforschung zuzuordnen, doch bewegen wir uns nahe der Schnittstelle zur klinischen Forschung und haben immer einen möglichen Nutzen für unsere Patienten im Blick. Somit verwenden wir Gewebe und Zellen aus verschiedenen klinischen Szenarien zur Validierung der von uns entwickelten Verfahren. Die folgende Originalarbeit beschreibt die Auswahl und Anwendung neuer DNA Sonden zum Nachweis von Aneuploidien für menschliche Gonosomen und Autosomen und zeigt deren Anwendung beispielsweise an Modellen aus den Bereichen der Präimplantationsdiagnostik, der Pränatalmedizin und der Onkologie.

2.5. Originalarbeit:

Bioinformatics Tools Allow Targeted Selection of Chromosome Enumeration Probes and Aneuploidy Detection [6]

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Abstract

Accurate determination of cellular chromosome complements is a highly relevant issue beyond prenatal/pre-implantation genetic analyses or stem cell research, since aneusomy may be an important mechanism by which organisms control the rate of fetal cellular proliferation and the fate of regenerating tissues. Typically, small amounts of

individual cells or nuclei are assayed by *in situ* hybridization using chromosome-specific DNA probes. Careful probe selection is fundamental to successful hybridization experiments. Numerous DNA probes for chromosome enumeration studies are commercially available, but their use in multiplexed hybridization assays is hampered due to differing probe-specific hybridization conditions or a lack of a sufficiently large number of different reporter molecules. Progress in the International Human Genome Project has equipped the scientific community with a wealth of unique resources, among them recombinant DNA libraries, physical maps and data-mining tools. Here, we demonstrate how bioinformatics tools can become an integral part of simple, yet powerful approaches to devise diagnostic strategies for detection of aneuploidy in interphase cells. Our strategy involving initial in-silico optimization steps offers remarkable savings in time and costs during probe generation, while at the same time significantly increasing the assay's specificity, sensitivity and reproducibility.

Keywords
molecular cytogenetics, fluorescence *in situ* hybridization, DNA probes, bioinformatics, data mining, interphase cells, aneuploidy, regenerating tissues

Footnote:
The results have been presented in part in poster format at the 2011 and 2012 Annual Meetings of the Histochemical Society held at the Marine Biological Laboratory, Woods Hole, MA.

Introduction

Nowadays, fluorescence *in situ* hybridization (FISH) plays a significant role in many research algorithms and became indispensible for routine clinical cytogenetic diagnostics (Anguiano et al. 2012; Bednarz et al. 2010; Ried et al. 1992; Tkachuk et al. 1991; Wallander et al. 2012). For example, in prenatal/pre-implantation genetic analyses (Cassel et al. 1997, Bucksch et al. 2012) or stem cell research (Hessel et al. 1996, Kwon et al. 2010; Weier et al. 2004), as a means of controlling the rate of fetal cellular proliferation at the fetal-maternal interface (Weier et al. 2005; 2010) or determining the fate of regenerating tissues, assessment of aneuploidy may be of substantial importance (Kodama et al. 2009; Kwon et al. 2010; Pujol et al. 2003). However, while other methods, such as chorionic villus sampling (CVS) with *in vitro* culture-based karyotyping of metaphase spreads have presented difficulties in detecting aneusomies because abnormal cells can arise in any portion of the placenta or fetal-maternal interphase, but might not be proliferating (Weier et al. 2005), we rely on chromosome-specific FISH assay to accurately determine the cellular chromosome complements in interphase cells.

Robust cytogenetic diagnosis depends on the availability of bright and specific FISH probes with carefully chosen fluorescent labels for multi-color, multi-target experiments (Avens et al. 2011; Bucksch et al. 2012; Hovhannisyan et al. 2012; O'Brien et al. 2010). Numerous commercially available probes presently serve the basic needs of the biomedical research community. But in order to be commercially viable, these probes have been restricted to high yield targets and typically are limited to a narrow choice of fluorochrome labels (Avens et al. 2011).

In many cases, seeking a conclusive answer to a well-defined, clinical question requires a custom nucleic acid probe that has to be specific and appropriately labeled,

yet it may not be available commercially. Therefore, these studies would require additional expertise in preparing target specific DNA and subsequent labeling with the hapten of choice (Baumgartner et al. 2006). Briefly, such tailored FISH experiments can be divided into several crucial steps or milestones: 1) choosing the appropriate genomic DNA target sequence, 2) sourcing or designing a suitable DNA probe, 3) devising a rational probe labeling/detection strategy, 4) optimizing wet lab procedures necessary to achieve optimal hybridization between the probe and the DNA target, 5) documenting the hybridization images and, finally 6) interpreting the results and putting them into context of clinical and histopathological findings (Wallander et al. 2012).

There is no doubt that information technology has lead to advances in most of the aforementioned steps. For example, composite image capture of several monochrome fluorescence images recorded at different wavelengths found its way into the routine clinical laboratory after multi-spectral digital imaging became affordable (Farkas et al. 1998; Levenson 2006; Wells et al. 2007). However, it has been difficult, time consuming and expensive to design and produce high quality DNA probes suitable for rapid, multiplexed chromosome enumeration or detection of chromosomal translocations (Fung et al. 1998, 2000; Greulich et al. 2000; Lu et al. 2009a; Weier et al. 1991b). Over the years, the research community has invested many man-hours in target-specific probe design, often duplicating each others efforts and unable to benefit from other groups' efforts with identical DNA targets (Devilee et al. 1988; Guan et al. 1996; Kievits et al. 1990; Lengauer et al. 1990; Vooijs et al. 1993; Vorsanova et al. 1986; Waye et al. 1987; Weier and Gray 1992; Weier et al. 1990b; Willard et al. 1983; Wolfe et al. 1985; Yurov et al. 1987).

Tailored bioinformatics-guided probe selection strategies provide powerful advantages in the first two fundamental steps of FISH-based chromosome counting. This database aided method is commonly referred to as 'data mining' and replaces time consuming and costly *in vitro* target acquisition experiments with a speedy and efficient *in silico* optimization, avoiding much of the time and resources spent in the wet lab. Laboratories experienced in FISH assays are therefore able to streamline the onerous process of optimizing the target DNA/probe match. The use of publically available, web-based bioinformatics tools (Kent et al. 2002) will also help expand the use of FISH into a technique that can be accessed by non-specialized and less established laboratories or groups with more constrained resources (Zeng et al. 2011).

In this communication, we report the development of a chromosome enumeration assay for use in reproductive studies that demonstrates the bioinformatics-guided FISH approach. In several distinct examples, we show how data mining strategies were used for target identification and probe selection. Probes were optimized to identify copy numbers of chromosome 10, as well as gonosomal genotyping (chromosomes X and Y). This assay development highlights the necessity of optimization and quality control steps throughout the process. Poor specificity complicates and limits the information that can be deducted from the hybridization result. We demonstrate how DNA sequence analysis can be used to predict probe performance. After selection and quality control experiments, the best BAC probes were applied in diagnostic scenarios including interphase and metaphase preparations from healthy volunteers, placental tissues as well as pathological samples such as a human cancer cell line. These typical diagnostic implementations demonstrate how the strategic introduction of bioinformatics tools into routine hybridization algorithms can save time and cost and improve the signal-to-noise-ratios, sensitivity, specificity and reproducibility of FISH experiments.

Materials & Methods

All procedures involving human subjects have been conducted under Human Subjects Use Protocols reviewed and approved by the UC Berkeley Institutional Review Board (UCB IRB).

Probe Development

Retrieval of X and Y Probe Target DNA Sequences for PCR amplificaitons
The nucleic acid sequence used for the Y chromosome-specific probe was defined in our previous studies using *in vitro* DNA amplification from the 3.6 kb pentanucleotide DNA repeat described by Nakahori et al. (DYZ1, Genbank accession number X06228) (Gray and Weier 1998; Nakahori et al. 1986). The annealing sites of primers WYR2 and WYR 4 (Table 1) were chosen to have a maximum of sequence deviation from the human satellite III DNA pentameric repeat consensus motif TTCCA, and to yield a 124bp DNA fragment by PCR (Gray and Weier 1999; Gray and Weier 1998; Nakahori et al. 1986; Weier et al. 1990b).

Name	Forward 5'-3'	Reverse 5'-3'	References
WXR1	tcgaaacgggtatatgctcacgtaaaa	WXR2	unpublished
WXR2	WXR1	aagacagtttcaaaactgctccatcaa	unpublished
WYR2	attccgtacgattccattccttttgaa	WYR4	(Weier, 1990)
WYR4	WYR2	gaatgtattagaatgtaatgaacttta	(Weier, 1990)

Table 1. Oligonucleotide sequences and PCR primer pairs used for DNA probe preparation

Blood samples from six normal human volunteers were used to validate the Y chromosome-specific PCR assay (Gray and Weier 1999; Weier et al. 1990a).

The oligonucleotide primers used to generate a X chromosome-specific DNA probe by PCR from genomic DNA are WXR1 and WXR2 (Table 1). They were defined based on the published DNA sequence of chromosome X-specific alpha satellite DNA (Waye and Willard 1985; Willard et al. 1983) and designed to yield a 124 bp DNA fragment. The PCR conditions and hybridization modalities have been described previously (Baumgartner et al. 2001; Robbins et al. 1995; Wyrobek et al. 1994).

DNA Amplification and Probe Biotinylation
Detailed methods have been described in our previous publications (Weier et al. 1994a; Weier et al. 1991a; Weier et al. 1990b). Briefly, genomic DNA was extracted from capillary blood from a male donor and DNA amplification was performed on the automated thermal cycling system designed and built in our laboratory. Unbound deoxynucleoside triphosphates were removed from the PCR solution by spinning the sample through a 1 mL Sephadex G-50 column (Pharmacia; Pleasant Hill, CA). Then, 5 µL aliquots of the PCR solution were re-suspended in 275 µL of biotinylation buffer (10 mM Tris-HCl, pH8.4, 1.5 mM $MgCl_2$, 50 mM KCl, dATP, dCTP, and dGTP, 0.2 mM each, 0.33 mM biotin-11-dUTP (Sigma-Aldrich; St. Louis, MO), 1.2 mM each primer) and 20 units of Taq polymerase (Perkin Elmer; Waltham, MA). The DNA solutions were then amplified for an additional 20 cycles to generate biotin-labeled DNA fragments, which were stored unpurified at -18°C in the freezer until used for FISH.

Retrieval of chromosome- specific probe information for the pericentromeric region of the target chromosomes using data mining and BAC-FISH methods
Database Searches for the Y-Chromosome specific DNA Probe
We screened the National Center for Biotechnology Information (NCBI) human genome nucleotide DNA database for homologous sequences. Execution of the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) querying the human genome database with the 27-nucleotide (nt) sequence 'ATTCCGTACGATTCCATTCCTTTTGAA' from position 3089-3115 of the human Y-specific 3564 bp repeat (Genbank accession number X06228) retrieved multiple hits. Parameters were chosen to identify clones with a range of nucleic acid homology (setting: 'Optimize for somewhat similar sequences', (BLASTn)).

Retrieval of probe information for the pericentromeric region of the X chromosome
We used the University of California Santa Cruz (UCSC) Genome Browser GRCh37/hg19, built February 2009, at the University of California, Santa Cruz, accessible at http://genome.ucsc.edu/ to identify bacterial artificial chromosome (BAC) clones with high satellite DNA content. The graphic user interface was set to display BAC end pairs and repeat DNA elements in the pericentromeric region of the human X chromosome, i.e., from position 58,232,531 to 61,922,800 bp.

Retrieval of Information for Probes in the Pericentromeric Region of Chromosome 10
Similar to chromosome X, we used UCSC Genome Browser GRCh37/hg19 (Kent et al. 2002), built February 2009 to identify BAC clones with high satellite DNA content around the pericentromeric region of human chromosome 10.

DNA Probe Preparation
The BAC DNAs were extracted from overnight cultures following an alkaline lysis protocol (Birnboim and Doly 1979) or using a ZR BAC DNA Miniprep Kit (Zymo Research; Irvine, CA). The isolation of high molecular weight BAC DNAs was confirmed on 1% agarose gels and quantitated by Hoechst fluorometry using a Hoefer TK 100 instrument (Hoefer; South San Francisco, CA). Probe DNAs were labeled with biotin-14-dCTP (Invitrogen; Carlsbad, CA), digoxigenin-11-dUTP (Roche Molecular Systems; Indianapolis, IN), Spectrum Orange-dUTP (Abbott; Abbott Park, IL), Spectrum Green-dUTP (Abbott) or Cy-5 –dUTP (*GE* Healthcare; Piscataway Township, NJ) by random priming using a commercial kit (BioPrime Kit, Invitrogen;) (O'Brien et al. 2010; Weier et al. 1995a). When incorporating fluorochrome-labeled deoxynucleoside triphosphates, the dTTP to dUTP ratio in the reactions was adjusted to 2:1 (Fung et al. 1998; Weier et al. 1994a; Weier et al. 1994b).

Cell Culture and Preparation of Metaphase Spreads
Control metaphase spreads were made from phytohemagglutinin stimulated short-term cultures of normal male lymphocytes according to the procedure described by Harper and Saunders (Harper and Saunders 1981). Fixed lymphocytes were dropped on ethanol-cleaned slides in a CDS-5 Cytogenetic Drying Chamber (Thermatron Industries, Inc.; Holland, MI) at 25°C and 45–50% relative humidity (Munné et al. 1996).
The S48TK cultures were established as described by Zitzelsberger et al. (Zitzelsberger et al. 1999). All procedures followed protocols approved by the Ethics Committee of the Bavarian Board of Physicians and the LBNL/UC Berkeley Committee on Human Research regarding use of surplus surgical tissues for research. In essence, S48TK lines

were obtained from the tumor tissue of a 14-year-old patient (7 years old at time of exposure to elevated levels of radiation) undergoing surgery at the Center for Thyroid Tumors in Minsk, Belarus, following the diagnosis of Hashimoto's thyroiditis and papillary thyroid cancer (PTC). Initial chromosome preparations were carried out after an *in vitro* culture of S48TK cells for 8–21 days. Later on, clones were isolated by limiting dilution and cultured for more than 20 passages. The clone number is indicated following the name of the primary line, S48TK. For example, S48TK6 and S48TK18 refer to clone 6 and clone 18 of cell line S48TK. After G-banding with Wright's staining solution, karyotypes were recorded according to the International System for Human Cytogenetic Nomenclature (ISCN, 2005). To further reduce heterogenicity, a second round of cloning by limiting dilution was performed on 96-well microtiter plates. Resulting clones were identified by adding the plate position to the parental clone name, i.e., line S48TK18C3 has been derived from line S48TK18.

Spectral Karyotyping (SKY) Analysis
The SKY analysis was performed as described previously (Fung et al. 1998, 2000; Garini et al. 1996; Schroeck et al. 1996; Weier et al. 2011; Zitzelsberger et al. 1999, 2001, 2002). Briefly, acetic acid:methanol (1:3 , vol.:vol.) fixed metaphase spreads were pretreated with RNAse A and pepsin, then fixed in freshly prepared 1% para-formaldehyde (FA), hybridized with a probe mix comprised of 24 chromosome-specific painting probes and detected following the probes manufacturer's instructions (Applied Spectral Imaging (ASI); Carlsbad, CA).

Tissue Section Preparation
Deparaffinization, slide pretreatment, and FISH hybridization procedures followed the published protocol (Rautenstrauss and Liehr 2002). Briefly, the mounted section was deparaffinized in 50 ml xylene in Coplin jars (two times 5 min each), rehydrated in an ethanol series (100%, 90%, 70%, 50%; 3 min each) and 0.9% NaCl solution (2 min x 2). If necessary, undesirable parts of the tissue and excess paraffin can be removed at this point by scratching it off of the slide with a scalpel.

As in a conventional FISH approach, a pretreatment of the slides with RNase and pepsin followed by postfixation with formalin-buffer is required to reduce the background (Liehr et al. 1995). Slides are then soaked in 2X SSC for 5 min at 21°C (in a 50ml Coplin jar on a shaker) (20X SSC is 3 M sodium chloride and 300mM tri-sodium citrate, pH 7.0). Slides are removed from the Coplin jar, and 100µl of RNase solution is added per slide and covered with a suitably sized coverslip (RNase solution: 50 µg/ml in 2X SSC). The slides are incubated in a humid chamber for 15 min at 37°C, then washed with 2X SSC for 3 min on a shaker, before a final wash with 1X PBS for 5 min. Slides are then pretreated with pepsin-buffer at 37°C for 10 min (without agitation) (pepsin buffer: freshly prepared 50-100µg/ml pepsin in 0.01 M HCl, prewarmed to 37°C). This is replaced with 1X PBS/$MgCl_2$ (5%(v/v) 1 M $MgCl_2$ in 1X PBS) and incubated at 21°C for 5 min with gentle agitation. Nuclei are postfixed on the slide surfaces by replacing 1X PBS/$MgCl_2$ with formalin-buffer (1.5 ml acid free formaldehyde in 50ml 1X PBS) for 10min (21°C, with gentle agitation). Formalin-buffer is replaced by 1X PBS for 2min (21°C, with gentle agitation). Finally slides are dehydrated by an ethanol series (70%, 85%, 100%; 3 min each) and air dried.

Fluorescence in situ Hybridization (FISH)
A 100µl aliquot of denaturation buffer was added to the slides (metaphase or interphase cell slides or deparaffinized tissue section slides) and covered with appropriately sized coverslips. Denaturation-buffer is 70%(v/v) deionized formamide, 10% (vol./vol.)

filtered double distilled water, 10% (vol./vol.) 20X SSC, 10% (vol./vol.) phosphate buffer. Phosphate-buffer is 1:1 mixture of 0.5 M Na_2HPO_4 and 0.5 M NaH_2PO_4, pH 7.0, and stored as aliquots at -20°C. Slides were incubated on a hot plate for 2-5 min at 75°C. The coverslips were removed immediately with forceps and the slides were post-fixed in 70% ethanol at 4°C to conserve target DNA as single strands. Slides were dehydrated in ethanol (70%, 85%, 100%, 4°C, 3 min each) and air dried.

For hybridization, 1 µl of either labeled DNA probe, 1 µl of salmon sperm DNA (10 mg/ml, 5 Prime – 3 Prime Inc.; Boulder, CO), 1 µl water, and 7 µl of the hybridization master Mix (78.6% formamide, 14.3% dextran sulfate in 1.43X SSC, pH 7.0) were thoroughly mixed and denatured at 76°C for 10 min. The final concentration in the hybridization mixture is 55% formamide (FA, Invitrogen; Carlsbad, CA), 10% dextran sulfate and 1X SSC, pH 7.0. About 2.5 µl of hybridization mixture was placed on the denatured samples, covered with a 12 mm circular coverslip, sealed with rubber cement and incubated at 37°C in a humid chamber (for metaphase and interphase slides: incubated for 16-20 hrs; for SKY analysis: incubated for 48 hrs; for deparaffinized tissue section slides: incubated for 72 hrs).

Post-Hybridization Washes and Detection of Bound Probes
After the rubber cement was carefully removed from the slides, the coverslips were allowed to slide off in 2X SSC at 20°C. Post-hybridization washes were performed as described and included two washes in 50% FA/2X SSC buffer at 43°C for 15 min each followed by two washes in 2X SSC at 20°C, each for 10 min (Kwan et al. 2009; Lu et al. 2009b). If labeled indirectly, after unspecific binding sites were blocked with PNM buffer (5% nonfat dry milk, 0.1% NaN_3 in PN buffer, that is 0.1M sodium phosphate buffer, pH 8.0, plus 0.05% NP-40) for 10 min, non-fluorescent probes were detected with either fluorescein-conjugated avidin DCS (Vector; Burlingame, CA) or anti-digoxigenin rhodamine (Roche; Indianapolis, IN), and two 2X SSC washes were used to wash away unbound antibodies. Finally, the slides were mounted with 4,6-diamino-2-phenylindole (DAPI) (0.1µg/ml) in antifade solution (0.1% p-phenylenediamine dihydrochloride (Sigma-Aldrich), 0.1X PBS (Invitrogen; Carlsbad, CA), 45 mM $NaHCO_3$, 82% glycerol (Sigma-Aldrich), pH 8.0) and coverslipped.

Image Acquisition and Analysis
Fluorescence microscopy was performed on a Zeiss Axioskop microscope (Zeiss, New York, NY) equipped with filter sets for observation of Cy5/Cy5.5, Texas red/rhodamine, FITC or DAPI (84000v2 Quad, ChromaTechnology; Brattleboro, VT). Images were collected using a CCD camera (VHS Vosskuehler; Osnabrueck, Germany) and processed using Adobe Photoshop® software (Adobe Inc.; Mountain View, CA) (Weier et al. 2011).

Results

Chromosome X and Y Specific Probe Selection by PCR Assay and Validation by FISH
The PCR product resulting from the PCR (primers WYR2 and WYR4) is approximately 124bp (gel picture not shown), as expected. The PCR product resulting from the PCR (primers WXR1 and WXR2) is 124bp. Both PCR products were labeled with biotin and used for *in situ* hybridization without further purification. Figure 1A shows the results of hybridization of the probe to the long arm of the Y chromosome.

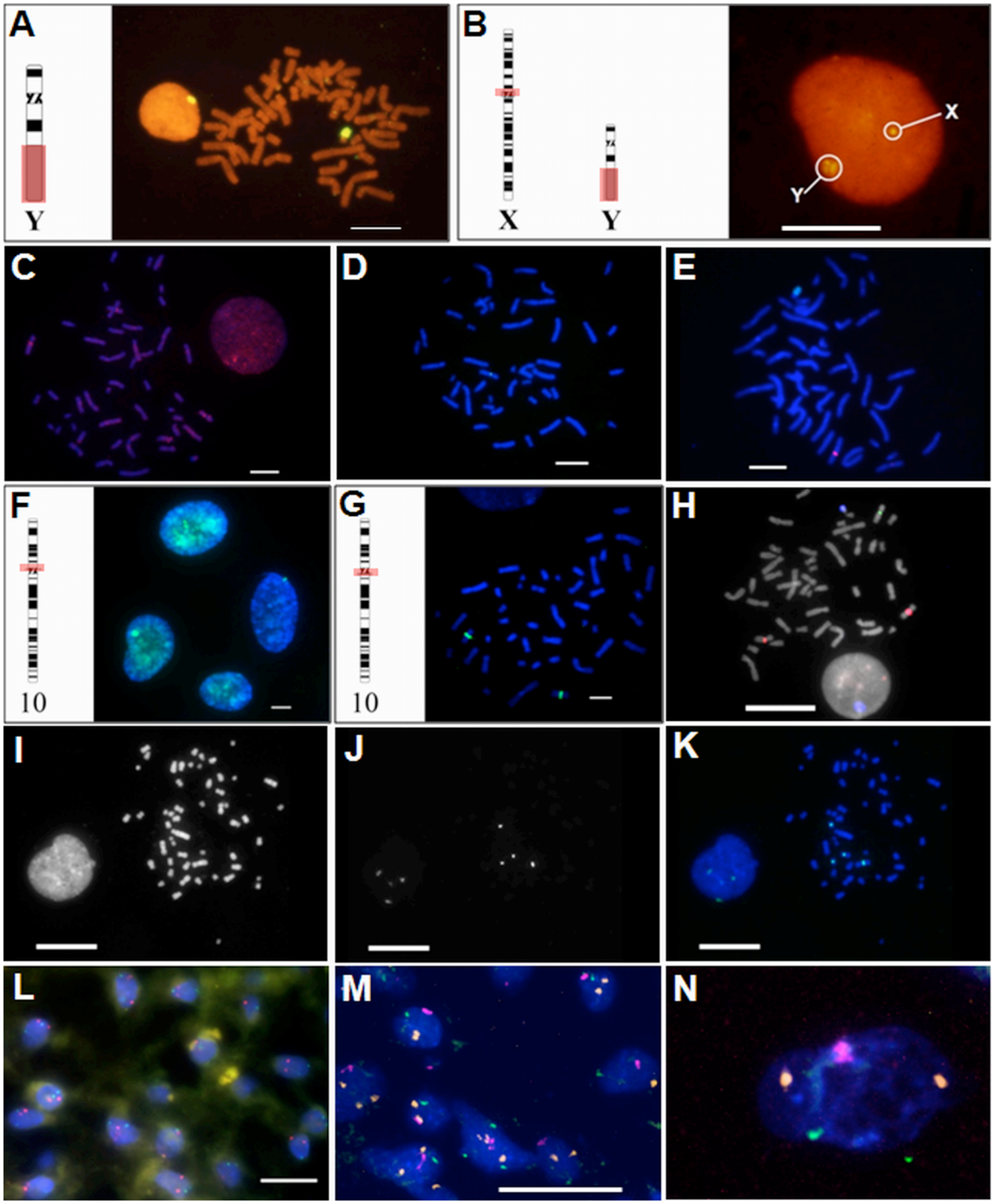

Figure 1. *In situ* hybridization results for selected DNA probes. Red shaded areas on the idiograms in A-B) and F-G) indicate the hybridization target sites. **A)** *In situ* hybridization using fluorescent probes for the highly repeated satellite III DNA segment (flanked by the PCR primers WYR2 and WYR4) shows bright signals on the long arm of the Y chromosome. **B)** Combining biotinylated *in situ* hybridization probes for the Y chromosome-specific DNA segment (large signal) with probes for X chromosome-specific DNA flanked by the PCR primers WXR1 and WXR2 (small signal) allows chromosome identification based of the size of the signal. **C)** A digoxigenin-labeled DNA probe prepared from BAC RP11-348g24 resulted in specific signals on the X chromosome in the presence of a significant amount of cross-hybridization to other chromosomes in metaphase spreads. High level of cross-hybridization also shows up in

interphase nuclei. **D)** In contrast to the result shown in C), a probe prepared from BAC RP11-294c12 bound almost exclusively to the centromeric heterochromatin of the human X chromosome. **E)** Combining differentially labeled DNA probes in a single hybridization experiment, the Y chromosomal target appears in green, while the X chromosomal centromeric repeat DNA is shown in red. **F)** *In situ* hybridization of an autosome-targeting DNA probe prepared from BAC clone RP11-96f8 shows multiple signals and high level of cross-hybridization in the interphase cell nuclei. **G)** The biotinylated probe prepared from BAC RP11-168p20 exhibits strong signals on both homologues of chromosome 10. **H)** A combination of three differently labeled probes was hybridized simultaneously. Chromosome 10 signals are shown in red; X chromosomal and Y chromosomal signals are shown in green and blue, respectively. **I-K)** The same probe as in G) (BAC RP11-168p20) was hybridized to the cell line S48TK18A6. Multiple signals indicate chromosomal abnormalities. I) shows DAPI picture, J) shows green avidin-FITC signal, and K) shows the superposition of both. **L-N)** A chromosome X probe (BAC clone RP11-294c12) labeled with biotin/avidin-FITC, a Cy-5 labeled chromosome Y probe (RP11-242e13) and a chromosome 10-specific probe prepared from BAC RP11-168p20 (labeled with Spectral Orange-dUTP) were hybridized onto a deparaffinized 12 week placenta tissue section. **L)** The chromosome 10 probe (red signals) and bound chromosome X probe (green signals) in this female placental tissue section. M-N) In our triple probe FISH experiment, the chromosome 10 probe shows orange signals, the chromosome X probe shows green signals, and the chromosome Y probe shows red signals in the male cell nuclei. (Size marker bars indicate 10 μm).

Figure 1B shows the combination of biotinylated probes for both Y- and X-chromosomes. The signals can be distinguished by size. Biotin-labelled probe detection was accomplished using avidin-FITC as described (Weier et al. 1990b). The chromosomes and cells were counterstained with propidium iodide (PI). Fluorescence from the hybridized probe appears yellow (resulting from the green of the avidin-FITC overlayed onto the red PI staining).
Even with similar probe concentrations the two labeled DNAs show drastically different hybridization specificities (Figures 1C and 1D).The digoxigenin-labeled probe prepared from BAC clone RP11-348g24 containing the interspersed repeats gave a strong signal on the X chromosome, but also unacceptably high levels of cross-hybridization on the autosomes (Figure 1C). In interphase nuclei,such high levels of cross-hybridization prevented identification of the X chromosome target. In contrast, hybridization of a biotinylated DNA probe prepared from BAC clone RP11-294c12 resulted in signals that localized exclusively to the pericentromeric region of the human X chromosome (Figure 1D). Figure 1E demonstrates that the highly specific DNA probes for chromosomes X and Y can be combined in dual-color multiplex hybridization experiments (Bednarz et al. 2010; Fung et al. 2001; Jossart et al. 1996). We labeled the Y chromosome-specific BAC clone RP11-242e13 directly with Spectrum Green-dUTP (green fluorescence) and the X chromosome-specific BAC clone RP11-294c12 with Spectrum Orange-dUTP (red fluorescence). Hybridization of this probe mixture gave strong, specific signals in metaphase as well as interphase cells (Figure 1E).

Chromosome X and Y BAC clone selection and validation
Chromosome Y and X BAC clone selection and validation was previously described in Zeng *et. al.* (Zeng et al. 2011, 2012). A chromosome specific 3.5kb satellite III DNA

repeat had been identified (Nakahori et al. 1986) in the q12 band of chromosome Y, for which we designed and tested various PCR primer sets (Zeng et al. 2011). The bacterial artificial chromosome clone RP11-242e13 (Genbank accession number AC068123) had the highest homology score when compared to the best performing primer (a 27 nucleotide (nt) primer). Clone RP11-242e13 actually contained 28 copies of a sequence with 85% homology to this primer within its 98295 bp insert.

For the X chromosome, we observed that chromosome-specific alpha satellite DNA repeats can be identified in BAC clones through database searches (Baumgartner et al. 2006). Based on this prior observation, we identified two BAC clones with large inserts that targeted the tandem DNA repeats in the heterochromatic pericentromeric region of the human X chromosome. As shown in a screen capture from the interface of the UC Santa Cruz Genome Browser (Figure 2) (Zeng et al. 2011), one clone (RP11-294c12) is essentially free of interspersed DNA repeats, such as short or long interspersed elements (SINEs, LINEs, respectively) and maps to X q11.1 and a second clone (RP11-348g24), which contains a few interspersed DNA repeats maps to chromosome X, band p11.1.

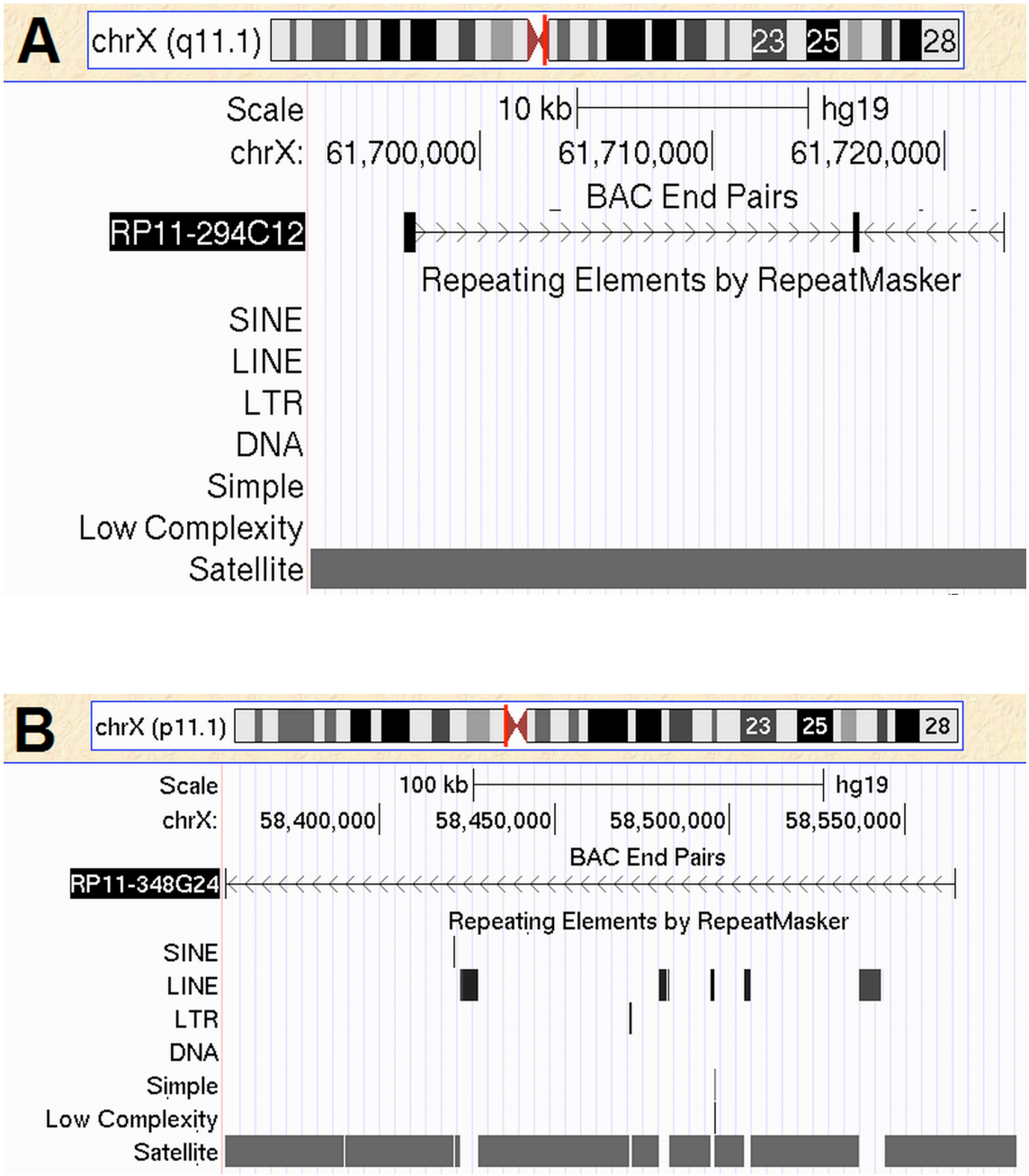

Figure 2. Datamining selection of FISH probes for chromosome X. Screenshots of the UCSC genome browser querry for BAC clones tagetting repeat rich regions of chromosome X. **A)** Clone RP11-294c12 has a target sequence within the pericentromeric heterochromatin of the q arm that is rich in satellite repeats whereas **B)** a clone (RP11-348g24) selected from the pericentromeric region of the p arm contains both satellite and non-chromosome specific repeats (LINEs, SINEs and LTRs).

BAC clones for the pericentromeric region of chromosome 10

Similar to the X Chromosome probe data mining search, we used the graphical user interface of the UC Santa Cruz Genome Browser and distinguished two BAC clones (Figure 3A) located on the pericentromeric region of Chromosome 10, band 10p11.1. One is RP11-96f8, containing several interspersed DNA repeats, which when

hybridized showed multiple signals and high level of cross-specificity in interphase nuclei of normal cells (Figure 1F).

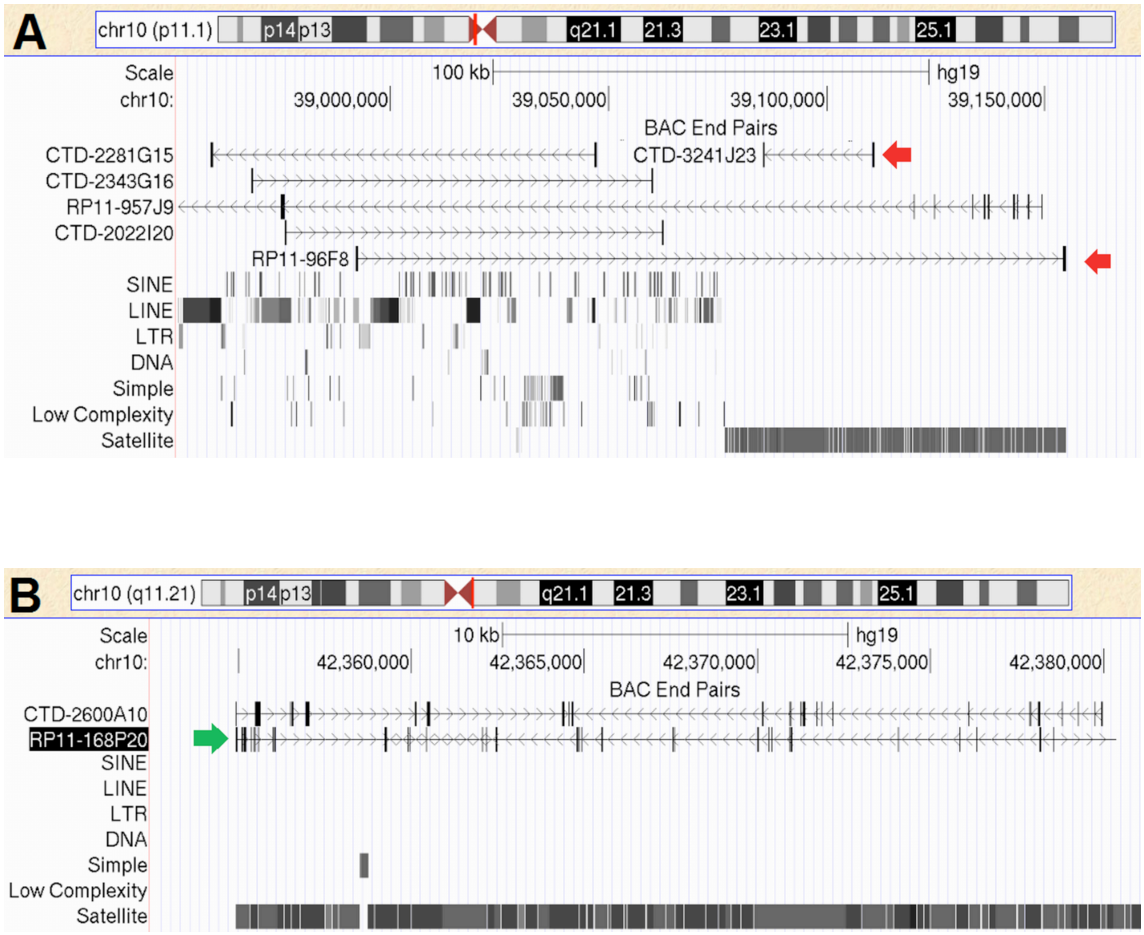

Figure 3. Data mining for probes specific for chromosome 10. A) Two probes selected for chromosome 10 (red arrows) contained both non chromosome-specific repeat regions (i.e., interspersed repeats such as SINE, LINE) and chromosome-specific (satellite) DNA repeats and produced poor hybridization results. **B)** A search in another repeat-rich region of chromosome 10 (q11.21) resulted in an alternative probe (green arrow) which produced the desired strong and specific signals in the presence of background signals.

However, the other clone CTD-3241j23 chosen contains no interspersed repeat DNA (Figure 3A), but shows weak to little hybridization signals on Chromosome 10, likely due to it's smaller size/coverage. In our search for a better candidate target, we turned to the other side of the pericentromeric region of chromosomes 10, band 10q11.21, and found a BAC clone RP11-168p20, which is composed of alpha satellite repeat DNA (of a pure single repeat sequence) and contains no LINES or SINES (Figure 3B). A biotinylated probe prepared from BAC clone RP11-168p20 gave a very strong and specific signal localized to the centromeric region of chromosome 10, with no observable cross-hybridization in metaphases of normal cells (Figure 1G).

Hybridization of probe RP11-168p20 to tumor cells reveals polyploidy
The same probe (prepared from BAC clone RP11-168p20) was hybridized to papillary thyroid cancer (PTC) cell line S48TK18, clone A6 (Weier et al. 2011; Weier et al. 2006; Zitzelsberger et al. 1999). A majority of cells showed four signals (Figure 4A).

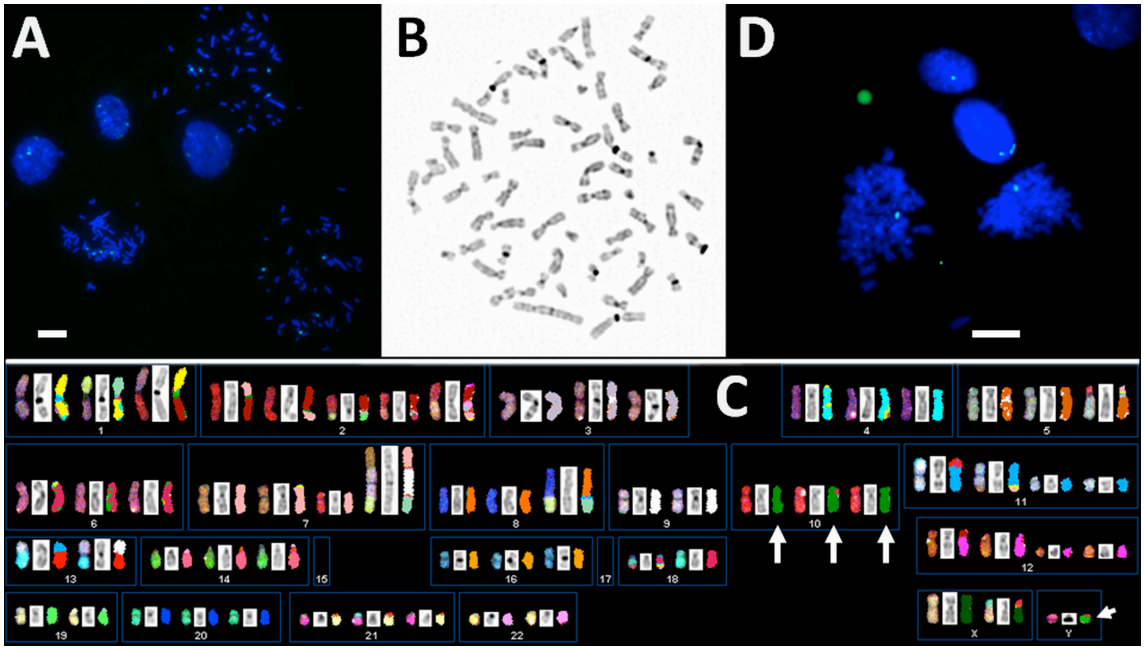

Figure 4. Hybridization of probe RP11-168P20 to tumor cells reveals polyploidy. **A)** Hybridization of a biotinylated probe to cells from the PTC cell line S48TK18, clone A6 shows cells with four signals. Please note the bright signals and absence of cross-hybridization on metaphase spreads. Some signals in interphase cells are not visible, likely due to being out of the focal plane of the image. **B-C)** Spectral Karyotype analysis of cell line S48TK6, clone C3. **B)** shows the inverted DAPI image of a metaphase spread. (Size marker bars indicate 10 μm). **C)** shows the SKY karyogram. The three large arrows point to the copies of chromosome 10 identified in this metaphase spread. The small arrow points to a small chromosome identified by SKY as the Y chromosome. **D)** Confirmation of the presence of Y chromosomal material via hybridization of a commercially available Y chromosome-specific DNA probe labeled with Spectrum Green (Vysis, Inc.).

Three independent analysts scored the signals and chromosomes in each of 40 metaphase spreads. The results can be grouped into two genotypes (Figure 5, Table 2).

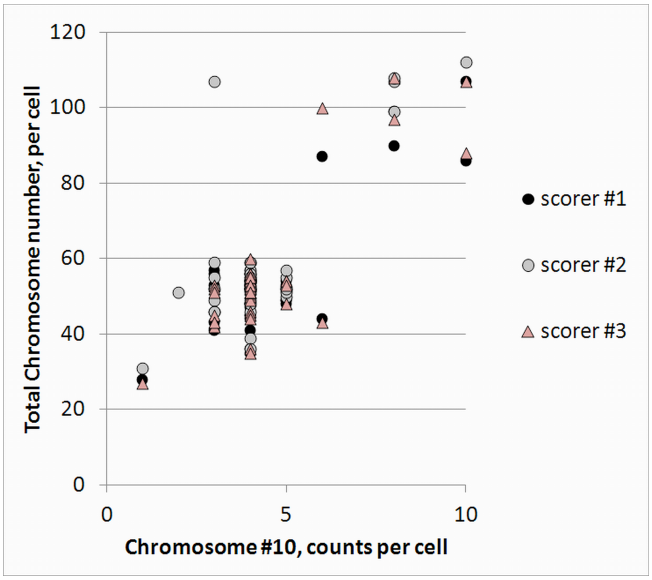

Figure 5. Culture genotype of chromosome 10 in a PTC cell line. Chromosome numbers and signal counts of the chromosome 10-specific DNA probe prepared from BAC RP11-168p20 hybridized to cell line S48TK18A6 (scored by three independent scorers).

	Group 1		Group 2	
	Average No. of Chromosomes	Average No. of Chr#10 signals	Average No. of Chromosomes	Average No. of Chr#10 signals
scorer #1	49	4	94	8
scorer #2	51	4	107	7
scorer #3	50	4	100	8

Table 2. Scoring RP11-168p20 (labeling chromosome 10) signals and total chromosome counts in thyroid cancer cell line S48TK18A6.

A large proportion of the metaphase spreads had on average 4 signals for chromosome 10 (out of a total of about 50 chromosomes per cell); while another smaller group had about 8 signals (out of a total of 100 chromosomes per cell, or double the chromosome count). S48TK6, clone C3 carries 3 copies of chromosome 10. A small Y chromosome was identified with SKY and confirmed by hybridizing a comercially available Y chromosome probe in interphase cells.

Hybridization of combined X-, Y-, and chromosome 10 probes onto deparaffinized 12 weeks placenta tissue section.
When a combination of three differentially labeled probes was hybridized onto deparaffinized placenta (12 week old) tissue sections on glass slides, individual signals could be distinguished. In slides made with female placental tissue (Figure 1L), only chromosome X and 10 signals were visible. Slides made from male placental tissue (Figure 1M-N) additionally showed signals from the Y chromosome-specific probe.

Discussion

About four decades after its inception, in situ hybridization has established itself firmly as an indispensable tool in the genetic characterization of cells in health and disease. While the initial, ground-breaking studies used isotopically-labeled nucleic acids probes (Pardue and Gall 1969; 1970), these techniques have mostly been replaced by assays involving non-isotopically labeled probes and detection by fluorescence microscopy (Manuelidis 1978).
Recent advances in bioinformatics have significantly facilitated the development of new algorithms to investigate genotypes for research or diagnostics. This contributes to our assessment that cytogenetic techniques and especially Fluorescence in Situ Hybridization, if combined with bioinformatic support tools, will be able to reach a new level of efficacy and user-friendliness.
When interrogating sample DNA for numerical and structural chromosomal abnormalities, it is of fundamental importance to make use of chromosome- or gene-specific nucleic acid probes that, once bound to their targets produce specific, unambiguous signals. BAC probes covering a majority of the human genome, and indeed DNA loci of interest in several other species, are now commercially available. These probes are tested, quality controlled and can easily be used in FISH hybridizations with a high success rate.
However, purchasing commercially available DNA probes comes at a huge expense when performing larger multi-target screening studies. Compounding this, probe composition remains almost always proprietary, thereby restricting the end user to applications specified by the probe manufacturer. Undisclosed addition of blocking reagents, a customary procedure in the preparation and 'copy protection' of single copy DNA probes, may furthermore interfere with multiplex assays.
Furthermore, commercial FISH probes for specific loci or centromeres are available with a limited array of distinct fluorochromes and cannot satisfy all of the possible label combinations required in specific multiplexed assays.
This shortcoming of commercially available probes increases with the ever expanding number of regions of interest and fluorochrome/detection modalities. Strategies for efficient in house target specific probe development are therefore a critical component of most *de novo* multiplexed FISH assays.

Bioinformatic platforms now allow the research community to share and summarize knowledge on target DNA sequences and probes. Running a publically available bioinformatics search tool, such as the Basic Local Alignment Search Tool (BLAST), against a database such as the National Center for Biotechnology Information (NCBI) human genome nucleotide DNA database, allows in-silico identification of appropriate target DNA sequences. Many hours of optimization experiments *in vitro* can be bypassed by spending a fraction of the time researching the optimal target/probe combination *in silico*.

We describe how the use of bioinformatic tools for chromosome X and Y BAC clone selection and validation convincingly demonstrates the power of this modified approach. From the onset we had a clearer understanding of the DNA target/probe interaction we could expect. For example, it could be forecast that clone RP11-294c12 would yield better specificity for chromosome X than clone RP11-348g24, due to the lack of non-chromosome specific repeat elements in its genomic target sequence (LINEs, SINEs and long terminal repeats (LTRs)). The lack of specificity of the later clone greatly reduces the ability to reliably count chromosomes in interphase cells. Prior *in silico* screening/optimization in this case would fast-track the identification of a reliable chromosome X enumeration probe. This optimization step would also greatly

shorten the efforts in developing multiplexed assays. If we had had the advantage of bioinformatic prescreening at our disposal when we designed our Chromosomal Rainbows (O'Brien et al. 2010) we could have saved vast amounts of time and expenses during probe selection, validation and optimization. Analogous to the example for chromosome X probe selection, we used *in silico* analysis to help predict the *in vitro* behavior of candidate probes for chromosome 10. As expected, the probe predicted with the greatest specificity and largest target area/brightest signal (RP11-168p20) yielded near-perfect *in vitro* hybridization results compared to a probe suspected to be suboptimal (RP11-96f8) (Baumgartner et al. 2006). In future selection strategies, such supoptimal probes could be omitted from any further *in vitro* testing.

The optimized chromosome 10 targetting probe (RP11-168p20) was then used to assay both normal and a cancer derived cell line (S48TK18, a PTC cell line that has been used extensively in our laboratories). Independent scoring by three different investigators gave highly corroborated results. This demonstrates the high quality chromosome enumeration probe selection that can be accomplished with the aid of bioinformatic tools.
We additionally used all three chromosome specific probes chosen with the help of *in silico* screening and *in vitro* testing in a typical diagnostic setting relevant to a clinical application in pre-natal diagnostics. The resultant tissue hybridizations provided clear, easily scorable chromosome enumeration images. A laboratory interested in investigating chromosomal status of placental tissue, or indeed any other tissue of interest, could make full use of the now publicly available bioinformatics resources and arrive at an applicable FISH based chromosome enumeration assay for their own purposes.
Through the combination of modern web-based tools, such as the UCSC genome web browser (http://genome.ucsc.edu/), the informatics resources at the Wellcome Trust Sanger Institute, Hinxton, UK, as well as the Resources for Molecular Cytogenetics, University if Bari, Italy, and the databases of commercial biotech companies offering specific high quality BAC probes such as Empire Genomics of Rochester, NY, it is now well within the capability of a basic cytogenetic laboratory to perform genotype investigations hitherto reserved to well funded and well staffed laboratories on the forefront of probe design and development.

In summary, bioinformatic techniques, including data mining, have not changed the fundamental principles of cytogenetic experiments. But they have provided a great tool for advancing the efficiency of such investigations, thereby making the technique more accessible to laboratories with less in-house expertise and funding, whilst simultaneously improving the process and outcome for all users.

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3. Diskussion

Personalisierte Medizin

Um zielführend personalisierte und stratifizierte Medizin praktizieren zu können bedarf es dreier Faktoren:

- Zum einen muss ein Verständnis dafür vorliegen, wo entlang des Genotyp - Phenotyp Kontinuums die individuellen Unterschiede liegen.
- Dann bedarf es eines Markers, um den individuellen Patienten einer distinkten Subgruppe zuordnen zu können.
- Schließlich müssen wir validierte Daten sammeln, die belegen, dass die Zugehörigkeit zu einer bestimmten Subgruppe therapeutische oder prognostische Relevanz hat.

Untersuchungen zu der Genotyp-Phenotyp Beziehung werden mit einer Vielzahl von Techniken vorgenommen. Hierzu bietet die Originalarbeit unter Abschnitt 2.2 eine umfassende Übersicht [3].
Zielgerichtete molekulargenetische Testmethoden erlauben es also, Untergruppen zu bilden in Patientenkohorten, die nominell an der gleichen Erkrankung leiden. Diese Untergruppen können sich zum Beispiel dahingehend unterscheiden, dass sie von einer Intervention besonders profitieren, auf eine Therapie nicht ansprechen oder bei einer etablierten Therapie mit schwereren Nebenwirkungen rechnen müssen.

Fluoreszenz-in-Situ-Hybridisierung und Molekulargenetik

Der erste grundlegende Durchbruch, welcher die hier beschriebenen molekulargenetischen Methoden erst ermöglichen sollte, war die Beschreibung der Doppel-Helix Struktur der Desoxyribonukleinsäure (Desoxyribose Nucleic Acid, DNA) durch Watson und Crick im Jahre 1953 [7]. Die im Hinblick auf die Fluoreszenz-in-Situ Hybridisierung wichtigste Eigenschaft der DNA ist ihre Zweisträngigkeit mit der engen Bindung komplementärer Basenpaare.

Die zytogenetische Technik der Fluoreszenz-in-Situ-Hybridisierung (FISH) wurde in den frühen 1980er Jahren entwickelt [8]. FISH basiert auf der Formation von stabilen

Hybriden zwischen Nukleinsäuren. Eine künstlich hergestellte Sonde aus DNA oder RNA hybridisiert mit Nukleinsäuresequenzen in den zu untersuchenden Zellen. Die Sonden können je nach Fragestellung mit fluorezierenden Molekülen markiert werden. Durch diese Fluoreszenzmarkierung können eine Vielzahl von unterschiedlichen Sonden verschiedenfarbig detektiert werden und somit gleichzeitig hybridisiert werden. Ein hochentwickeltes Beispiel hierzu ist in meinem Artikel "'Chromosomal Rainbows' Detect Oncogenic Rearrangements of Signaling Molecules in Thyroid Tumors" beschrieben, welcher im Anhang zu dieser Habilitationsschrift zur Verfügung steht [9]. Diese Vielfarbigkeit ist der entscheidende Vorteil gegenüber den ursprünglichen radioisotopischen Markierungen aus der Anfangszeit in den sechziger Jahren des 20. Jahrhunderts [10].

Nach Jahren der Planung began im Oktober 1990 das Humane Genom Projekt (HGP). Es war zu seiner Zeit das größte und aufwendigste Forschungsprojekt der Menschheit, mit einem Budget von über 3 Billionen US Dollar für den öffentlich unterstützten Beitrag. Bekanntermassen gab es ab 1998 auch eine private, kommerzielle Gruppe, die im Wettstreit mit den öffentlichen Forschern sequenzierte. Im April 2003, zwei Jahre früher als vorhergesagt, wurde ein komplettes haploides Genom vorgestellt. In 2006 wurde in einer Sonderausgabe der Zeitschrift 'Nature' das letzte voll sequenzierte Chromosom veröffentlicht [11].
Dieses Projekt, welches somit 16 Jahre intensivsten Forschungsaufwandes von mehreren Teams bedurfte, wäre mit heutiger Technologie in wenigen Wochen und zu einem Bruchteil der Kosten zu leisten. Diese erstaunliche Entwicklung der Technologien und Techniken beruht natürlich zu weiten Teilen auf den Erkenntnissen welche während des HGP gewonnen wurden.
In den folgenden Jahren wurden die Ergebnisse geordnet und viele weitere Erkenntnisse hinzugefügt. Mit diesen Daten wurden wertvolle Resourcen geschaffen, welche überwiegend kostenfrei zur Verfügung gestellt werden und weitergehende Forschung ermöglichen. Ein gutes Beispiel hierfür ist der Genome Browser der University of California Santa Cruz (UCSC). Am 7. Juli 2000 veröffentlichte die UCSC Bioinformatik Gruppe den ersten funktionierenden Entwurf des Browsers im Internet. Die wissenschaftliche Gemeinde lud allein in den ersten 24 Stunden eine halbe Trillionen Bytes an Informationen vom UCSC Genom Server herunter. Diese ersten 24

Stunden waren das erste Mal, dass kostenloser und unbeschränkter Zugang zu der Sequenz des Bauplans der menschlichen Spezies möglich war.

DNA Sonden können verschiedentlich konstruiert werden.
Sogenannte 'Whole Chromosome Painting Probes' sind Sonden welche ein ganzes Chromosom markieren können. Dies ist hilfreich um inter-chromosomale Translokationen in Metaphase zu untersuchen [12, 13].
Intra-chromosomale Rearrangements können mit Chromosom-Band spezifischen Sonden sowohl in Metaphase, als auch in Interphase untersucht werden [14, 15].
'Locus-specific Probes' binden an kleinere, Gen- oder Locus-spezifische Regionen und sind auch in Hybridisierungen auf Nukleinsäuresequenzen in Zellkernen in Interphase gut zu differenzieren [9, 16, 17].

FISH hat weite Verbreitung in Forschungslaboren gefunden. Die Anwendung im klinischen Umfeld wurde bislang durch die limitierte Selektion von kommerziell verfügbaren, behördlich zugelassenen Sonden stark beschränkt. Im klinischen Alltag etabliert hat sich FISH vornehmlich in der Onkologie, der Präimplantationsdiagnostik und in der klinischen Genetik im engeren Sinne [16, 17].
DNA Sonden zu entwickeln, die in vielfarbigen, sogenannten 'multiplex assays' zuverlässig funktionieren war bislang kostspielig und bedurfte einer aufwendigen Infrastruktur und Spezialwissens.
Um diese Limitierung zu überwinden, hat unser Labor verschiedene Studien veröffentlicht, die die Nutzung der fundamental hilfreichen, öffentlich zugänglichen Resourcen des Humanen Genom Projektes, zum Beispiel physikalische Gen-Karten und rekombinante DNA Bibliotheken, veranschaulichen. Somit gewinnen auch weniger spezialisierte Labore Zugang zu dieser leistungsstarken Technik. Routinemäßige Diagnostik und größere Studien können in Zukunft auch von kleineren, klinischen und nicht-akademischen Laboren kostengünstig und zuverlässig durchgeführt werden.

Die Originalarbeiten

Die in Abschnitt 2.1 beschriebenen Unterschiede in den Entzündungsreaktionen einzelner Patienten sind, nicht zuletzt auf Grund der großen Komplexität des Immunystems und der Entzündungskaskade, noch nicht in ihren genetischen Ursachen geklärt worden. Durch Untersuchung in der Genomik, Protenomik und Metabonomik wird in verschiedensten Arbeitsgruppen versucht, ein umfassenderes Verständnis zu gewinnen, welche Faktoren die Qualität und Quantität einer Entzündungsreaktion bedingen. Eventuell eröffnen sich im Zuge dieser Untersuchungen auch mögliche Ansatzpunkte für eine therapeutische Intervention zur Modifizierung der ungewünschten Aspekte der Entzündungsreaktion. Die unerwarteten Ergebnisse der vorliegenden klinisch-deskriptiven Studie belegen, wie limitiert unser Verständnis der Auslöser und Multiplikatoren des angeborenen Immunsystems noch ist.

Die in den darauf folgenden Abschnitten vorgestellten Weiterentwicklungen der molekulargenetischen FISH Technik bemühen sich um die Verbesserung der grundlegenden Algorithmen und technischen Möglichkeiten zur zielgerichteten Gestaltung molekularer Marker für bekannte genetische Subgruppen, sowie der Beschreibung neuer Polymorphismen.
Hierbei konzentrieren wir uns auf die Herstellung von neuartigen DNA Sonden zur 'Enumeration' von Chromsomen und Ploidie Feststellung in Interphase Zellkernen und Metaphase Preparationen. Die Beantwortung solcher Fragestellungen bilden die weit verbreitesten Anwendungen der Technik in Forschung und Klinik. Die Mehrzahl dieser 'Chromosome Enumerator Probes' (CEPs) genannten Sonden zielen auf vielfach reiterierte, in Tandem wiederholte DNA Sequenzen, so dass viele Kopien der eher kleinen Sonde an die Zielsequenzen hybridisieren. Diese konzentrieren sich häufig auf eine dicht lokalisierte Chromosom Region. Es bestehen verschiedene Möglichkeiten der Isolation und Purifikation dieser Sonden und in den Materialien und Methoden Teilen der Abschnitte 2.3 bis 2.5 werden diese näher beschrieben oder entsprechend zitiert.

Dass solche Marker prognostische und therapeutische Relevanz haben können, ist in der klinischen Onkologie bereits vielfach demonstriert. Die Verwendung molekulargenetischer Marker zu Verbesserung der Patientenversorgung in nicht-onkologischen klinischen Fächern steht erst am Anfang, hat jedoch großes Potential.

In der im Abschnitt 2.3 vorgestellten Arbeit "Data Mining Empowers the Generation of a Novel Class of Chromosome-specific DNA Probes" wird das enorme Potential des 'Data Mining' diskutiert [4]. Diese Technik wird genomische Untersuchungen grundlegend verändern und die neuen Möglichkeiten, nicht zuletzt in Verbindung mit NexGen Daten, werden ausgeführt.

In Abschnitt 2.4 führt die Arbeit mit dem Titel "Bioinformatic Tools Identify Chromosome-Specific DNA Probes and Facilitate Risk Assessment by Detecting Aneusomies in Extra-embryonic Tissues" diesen Gedanken fort [5]. Neben der weiteren Beschreibung, wie die Bioinformatik in neue Algorithmen und Strategien zum Sonden-Design und der Validierung der Marker eingebunden werden kann, vertieft diese Publikation die Diskussion über die Rolle, die solche Sonden in der Risikoabschätzung in Forschung und Klinik spielen können. Weitere Ressourcen zur in-Siliko Sondenentwicklung werden aufgeführt.

Die Arbeit "Bioinformatics Tools Allow Targeted Selection of Chromosome Enumeration Probes and Aneuploidy Detection" in Abschnitt 2.5 schliesslich entwickelt diesen Ansatz noch weiter und unterstreicht die Pragmatik der von uns entwickelten Algorithmen und wie diese in Forschung und Klinik auch von weniger spezialisierten Laboren angewandt werden können [6]. Hier wird eine Technik mit enormem Potential einer breiteren Anwenderschaft zugänglich gemacht. Somit werden die Limitierungen des Angebots kommerziell erhältlicher Sonden überwunden.

Da die Hybridisierungs-Technik auf der Komplementarität von Nukleinsäuresequenzen beruht, kann dank der in der Folge des Humanen Genom Projektes geschaffenen Datenbanken, ein wichtiger Teil der Experimente in-Siliko, also virtuell, durchgeführt werden, statt kosten- und zeitaufwendig in-Vitro, also im Labor. Bei bekannter Ziel- oder Sondensequenz kann durch Abgleich komplementärer Nukleinsäuresequenzen in den vorhandenen Datenbanken eine zuverlässige Vorhersage zur Hybridisierungsqualität in-Vitro gemacht werden.

Eine Limitation der FISH Technik liegt in der Beschränkung der Untersuchungen auf die genetische Information. Die genaue Beschreibung des vorhandenen genetischen Codes besagt in sich noch nichts über die Aktivität dieser genetischen Region.

Transkription und Translation, alle weiteren Schritte entlang der Genotyp zu Phenotyp Expression, bedürfen weiterer Untersuchungen.
Neuerdings gibt es weitere Entwicklungen der FISH Technik, die auf mRNA-Hybridisierungen beruhen und somit den qualitativen, quantitativen und 3-dimensionalen Nachweis von Gen-Expression ermöglichen. Traditionelle Gen-Expressions-Studien bedienen sich einer Vielzahl etablierter Assays, insbesondere Blots. So kann mRNA mit Northern Blots oder PCR Techniken nachgewiesen werden. Western Blots und Eastern Blots sind der Standard zum Nachweis von Proteinen und ihren Modifikationen. Es haben sich vernetzte Forschungsgebiete, zum Beispiel Protenomics und Metabonomics herausgebildet, um entlang des gesamten Genotyp zu Phenotyp Pfades Ursachen und Wirkungen untersuchen zu können. Wenn Zusammenhänge robust nachgewiesen wurden, muss dies nicht in jedem einzelnen klinischen Fall wiederholt werden, sondern es können genetische Marker etabliert werden, die dann Dank der Fortschritte der FISH Technik und neuerer Sequenzierungstechniken breitere Anwendung erlauben. In vielen Fällen reichen einfache Blutabnahmen, oder die Gewinnung von Biopsie-Geweben um die notwendigen Untersuchungen in Interphase durchführen zu können. Somit kann die FISH Technik einen wertvollen Beitrag zur Diagnostik im Hinblick auf individualisierte Therapieempfehlungen leisten. Diese Methode ist praktikabel und für den Patienten nicht zusätzlich belastend.

Klinische Relevanz und Bezug zur Perioperativen Medizin und Anästhesie

Die Stiftung Personalisierte Medizin beschreibt folgende Bestandsaufnahme [1]:
"2012 sind für 18 Wirkstoffe zur Behandlung von acht Krankheitsbildern eine obligatorische therapiebegleitende Diagnostik vorgeschrieben. [So ist bei...] - HIV (Test auf CCR5-trope Viren), myeloische Leukämie mit den Unterformen chronisch (Test auf Philadelphia Chromosom) und akut promyelozytär (PML/RAR-alpha Gen), akute lymphatische Leukämie (Test auf Philadelphia Chromosom), Brustkrebs (Test auf HER2 Protein) , nicht-kleinzelliger Lungenkrebs (Test auf EGFR-Protein), Darmkrebs (Test auf Wildtyp-Gen KRAS), Magenkrebs (Test auf Wildtyp-Gen KRAS) und Malignes Melanom (Test auf BRAF V600E) - eine molekulargenetisch basierte Diagnostik zwingend vorgeschrieben. In der Behandlung der sieben Krebserkrankungen kommen zehn biopharmazeutische Wirkstoffe, davon drei

monoklonale Antikörper (Cetuximab, Panitumumab und Trastuzumab) und sechs Kinasehemmer (Dasatinib, Erlotinib, Gefitinib, Imatinib, Nilotinib und Vemurafernib) zur Anwendung. Bei HIV sind es der CCR5-Antagonist Maraviroc und der Nukleosidanaloge Reverse-Transkriptase-Hemmer (NRTI) Abacavir."

Doch auch im Bereich weit verbreiteter, sogenannter 'Blockbuster' Therapien sollte eine personalisierte Therapie eine größere Rolle spielen. Dies wird im Folgenden anhand des Beispiels des 'Angiotensin Converting Enzym' (ACE) Genotyps und der Therapie mit ACE Hemmern diskutiert.
Montgomery et al. beschrieben 1999 den ACE Gen Polymorphismus und erkannten dessen Auswirkungen auf Belastbarkeit und Leistungsfähigkeit des menschlichen Körpers [18]. Es existieren zwei Allele: Insertion (I) und Deletion (D). Der Polymorphismus bedingt messbare Unterschiede nicht nur im Labor in der Aktivität des Renin-Angiotensin Systems, sondern auch bei verschiedenen Beispielen für menschliche Höchstleistungen. So ist das 'I' Allel wiederholt mit verbesserter Ausdauer-Höchstleistung, wie zum Beispiel beim Triathlon, in Verbindung gebracht worden. Das 'D' Allel hingegen bedingt Vorteile für Kraft-Höchstleistungen, so zum Beispiel bei Sprint-Diziplinen im Schwimmsport. Zudem ermöglicht der I/I Genotyp eine verbesserte Anpassung an eine hypoxische Umgebung bei Besiedlung oder Eroberung von extremen Höhen [19].
Bei der Betrachtung einzelner Organe, bedingt der ACE Genotyp zum Beispiel unterschiedliche Veränderungen der linksventrikulären Muskelmasse als Reaktion auf Belastungen: D/D Genotypen zeigen eine überproportionale Zunahme der Muskelmasse, während I/I den geringsten Musklemassenzuwachs hervorbringen.

Die klinische Wirksamkeit und optimale Dosierung von ACE Hemmern wurde in großen Studien untersucht und beschrieben. In einer Metaanalyse von Studien mit insgesamt 158.988 Patienten wurde die Wirksamkeit von ACE Hemmern und Angiotensin Rezeptor Blockern bezüglich der Endpunkte Blutdrucksenkung und Mortalität beschrieben [20]. In diesen Studien wurde keinerlei Unterscheidungen nach dem ACE Genotyp vorgenommen. Das lässt vermuten, dass sich der Therapieerfolg und die Dosis nach dem größten gemeinsamen Nenner definiert und nicht nach der Maximierung von individuellem Nutzen. Für den einzelnen Patienten könnte es jedoch von fundamentalem Vorteil sein, eine ACE Hemmer Therapie an seinen individuellen

ACE Genotyp anzupassen [21]. Damit dies in größeren Studien und gegebenenfalls im klinischen Alltag möglich wird, bedarf es robuster, kosteneffizienter Methoden, den individuellen Genotyp zu bestimmen. Ich postuliere, dass die FISH Technik hierbei eine wichtige Rolle zu spielen hat.

Nach derzeitigen Schätzungen enthält das menschliche Genom etwa 25.000 Gene. Bei einem Bruchteil dieser Gene verstehen wir bislang alle physiologischen und pathophysiologischen Funkionen. Mit zunehmendem Verständnis werden auch die Kandidaten, welche als sinvoller molekulargenetischer Marker für Therapie oder Prognose verwendet werden können, in Anzahl und Signifikanz zunehmen.
Der Gewinn und die Verwertung dieser neuen Informationen über einen einzelnen Patienten bringt komplexe ethische und datenschutzrechtliche Herausforderungen mit sich. Diese Herausforderungen bedürfen der eingehenden Betrachtung und Diskussion. Dies geht über den Rahmen der vorliegenden kumulativen Habilitationsschrift hinaus. Der Fokus hier liegt auf der Beschreibung der Weiterentwicklung methodischer Möglichkeiten molekulargenetischer Diagnostik.

In vielen Institutionen, so auch in unserer, entwickelt sich die Anästhesie und Intensivmedizin zuhnehmend zum Fachbereich der 'Perioperativen Medizin' [22]. In dieser erweiterten Auslegung der Aufgaben der Anästhesie ist der Bezug zu den hier vorgestellten Forschungsergebnissen leicht nachzuvollziehen.
Gerade bei der Optimierung eines Patientenpfades von der Diagnose bis zur Rehabilitation werden die Vorteile der Personalisierung / Individualisierung der Interventionen sowie die Stratifizierung des individuellen Risikos zu spürbaren Vorteilen für die Patienten führen.
Eine Therapie mit ACE Hemmern ist hier ein gutes Beispiel. Wenn es um die Optimierung von kardiovaskulären Risikofaktoren vor einer elektiven großen Operation geht, oder um die optimale Therapie nach einer Herzoperation, ist die Kenntnis des ACE Genotyps von großem Vorteil.
Auch in einer Situation in der ein Patient mit Lungenversagen invasiv beatmet werden muss, wäre es von Vorteil die individuelle Hypoxieresistenz dieses Patienten abschätzen zu können, um übermäßig agressive und somit auf längere Sicht schädliche Beatmungsparameter minimieren zu können.

In der Anästhesie im engsten Sinne gibt es offensichtliche Anwendungsgebiete für mögliche molekulargenetische Algorithmen in der Zukunft.
Da die Anästhesie Polypharmazie routinemässig anwendet, ist das wachsende Forschungsgebiet der Pharmakogenetik hier von besonderer Bedeutung. Individuelle Reaktionen und Sensitivitäten auf die von uns verabreichten Medikamente nicht erst im Nachhinein beurteilen zu können, würde zahlreiche Nebenwirkungen und Über- oder Unterdosierungen verhindern.
Eine seltene, und seltener werdende, Komplikation der Anästhesie ist die Maligne Hyperthermie (MH). Diese potentiell tödliche Reaktion auf bestimmte Triggerstoffe ist genetisch bedingt und tritt familiär gehäuft auf.
Der Screening-Test ist derzeit der sogenannte in-vitro-Muskel-Kontraktions-Test. Ist dieser positiv, so wird der Patient und seine Blutverwandschaft genetisch untersucht [23].
Bislang konnten 6 verschiedene Genorte auf verschiedenen Chromosomen mit MH in Verbindung gebracht werden. Bei dieser Heterogenität bedarf es flexibeler, individuell anpassbarer genetischer Tests, aus denen dann für das weitere Screening aussagekräftige Marker entwickelt werden können.
Über 80% der MH Patientenweisen eine Veränderung im RYR-1 Gen auf Chromosom 19 auf. Bei Punktmutationen bieten sich PCR und Sequenzierungs-basierte Techniken an. Bei den bekannten Insertionen und Deletionen hingegen lassen sich mit Hilfe der Bioinformatik und der hier beschriebenen Algorithmen schnell valide Marker entwickeln.

Durch die enormen Fortschritte der Sequenzierungstechnik findet eine parallele Entwicklung statt. Man spricht von dem 1000-Dollar-Genom. Gemeint ist, dass es absehbar scheint, für lediglich $1000 die relevanten Regionen eines beliebigen menschlichen Genoms sequenzieren zu können. Das ist im Verhältnis zu den oben genannten 3 Billionen Dollar öffentlichen Geldes plus den vielen hundert Millionen Dollar privaten Kapitals, welche zur ersten Sequenzierung eines menschlichen Genoms investiert werden mussten, eine unglaubliche Entwicklung.
Selbst wenn eine Genom-Sequenzierung als Routineuntersuchung finanzierbar würde, gibt es noch zahlreiche ethische und datenschutzrechtliche Fragen zu klären. Die Folgen, welche das Wissen um ein persönliches Risiko, das unter Umständen nie, oder viele Jahre später zu einer Symptomatik führen könnte, auf einen Patienten haben kann,

sind unüberschaubar. Neben der psychologischen Belastung um solch ein Wissen gibt es auch praktische Überlegungen, etwa bezüglich Kranken- und Lebensversicherungsfragen.
Läge diese Information über einen Patienten jedoch vor, so könnte eine Vielzahl von bekannten Marker-Nukleinsäure-Sequenzen in kürzester Zeit in-Siliko, also im Computer, gegen das Patienten-Genom abgeglichen werden und eine umfassendere und prospektive Profilierung wäre möglich. Eine mit eingehender Beratung einhergehende Aufklärung über ein persönliches Risikoprofil bietet einem Patienten Möglichkeiten, modifizierbare Risikofaktoren gezielt zu beeinflussen, sowie Prioritäten und Planung für sein Leben zu überdenken.

Schwerpunkte und Prioritäten der Forschung

Abschließend ist festzuhalten, dass das hier besprochene Forschungsgebiet sich vollständig in dem Aktionsfeld 2 (Die Forschungsherausforderung: Individualisierte Medizin) des Rahmenprogramms Gesundheitsforschung der Bundesregierung 2011-2014 verorten lässt [24]:
"Das Verständnis grundlegender Krankheitsmechanismen wächst, eine auf die individuellen Bedürfnisse und Voraussetzungen zugeschnittene Medizin wird greifbar. Damit rückt auch das Ziel näher, ein selbstbestimmtes Leben im Alter bei gutem Gesundheitszustand zu ermöglichen. Die Bundesregierung unterstützt deshalb die Entwicklung von Diagnostika und Therapeutika und spannt in der Förderung den Bogen entlang des Innovationsprozesses von der lebenswissenschaftlichen Grundlagenforschung über die präklinische und klinische und klinisch-patientenorientierte Forschung bis zur Marktreife."

Ich freue mich daran mitzuarbeiten, dass unsere Forschungsergebnisse sich in den nächsten Jahren in spürbaren Vorteilen für unsere Patienten bemerkbar machen.

4. Zusammenfassung

In unserem Bestreben, jedem einzelnen Patienten die optimale Therapie zukommen zu lassen, gilt es die Evidenz-basierte Medizin dahingehend weiterzuentwickeln, dass Studienergebnisse zukünftig die genomischen Subpopulationen der Studienkohorte erfassen und mit 'Outcome' Daten korrelieren. Dann können Patienten, die unsere Hilfe suchen, idealerweise mit wenig aufwendigen Schnelltests über ihre genomischen Risikofaktoren, sowie potentielle therapeutische Angriffsstellen informiert werden und wir können eine rationale Therapieempfehlung abgeben.
In diesem Sinne wurden in der vorliegenden Arbeit Weiterentwicklungen molekulargenetischer Techniken vorgestellt. Diese Weiterentwicklungen basieren zu einem großen Teil auf der Ausschöpfung des Potentials moderner Bioinformatik.
Um im klinischen Alltag das Leben von Patienten positiv zu beeinflussen, müssen diese Techniken mit einfachen Mitteln reproduzierbar sein. Des weiteren dürfen Kosten und Zeitaufwand nicht einer breiteren Anwendung im Wege stehen.
Die Fluoreszenz-in-Situ-Hybridisierung hat ihre Praxistauglichkeit in der Onkologie schon unter Beweis gestellt. Die durch FiSH gewonnenen Informationen sollten auch bei nicht-onkologischen Krankheitsbildern, wie zum Beispiel in der kardiovaskulären Medizin, Beachtung und Anwendung finden.

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ERKLÄRUNG

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,

- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,

- mir die geltende Habilitationsordnung bekannt ist.

Berlin, 12/12/12

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Ben O'Brien