Aus dem Experimental and Clinical Research Center, Campus Buch,
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DISSERTATION

Functional analysis of the WDR66 gene

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von

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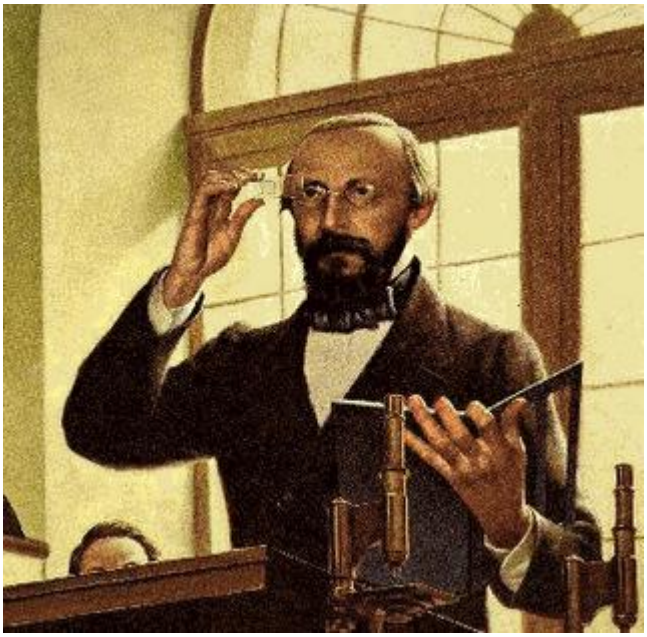
獻給我親愛的父母

For my Mother and Father



Die Ärzte sind die natürlichen Anwälte der Armen, und die soziale Frage fällt zu einem erheblichen Teil in ihre Jurisdiktion.

Rudolf Ludwig Karl Virchow

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LIST OF ABBREVIATIONS, SYMBOLS AND DIMENSIONS

℃	- Degree Celcius
DNA	- Deoxyribonucleic acid
ESCC	- esophageal squamous cell carcinoma
FBS	- Fetal Bovine Serum
5-FU	- 5-Fluorouracil.
GAPDH	- Glyceraldehyde-3-phosphate dehydrogenase
kDa	- kilo Dalton
LMD	- Laser microdissection
LES	- lower esophageal sphincter
n	- Number
NTC	- non treatment control
OCLN	- Occludin
PBS	- Phosphate buffered saline
qRT-PCR	- Quantitative real-time polymerase chain reaction
RIN	- RNA integrity number
RNA	- Ribonucleic acid
siRNA	- small interfering RNA or short interfering RNA
STS	- Staurosporine
UICC	- *Union Internationale Contre Le Cancer*

UES - upper esophageal sphincter

VIM - Vimentin

WDR66 - WD repeat-containing protein 66

1. SUMMARY

Esophageal cancer has long been considered one of the deadliest malignancies and considerable controversy has surrounded its management. Esophageal cancer patients have a dismal prognosis, because of the late presentation of patients with this disease and the technical difficulty of an adequate surgical resection in the presence of advanced local and regional involvement. The most common histological type is esophageal squamous cell carcinoma (ESCC), which is distributed throughout the length of the esophagus. In order to achieve a more tailored therapy and, consequently, improved prognosis, we studied gene expression profiles of ESCC in comparison to that of normal esophageal squamous epithelium with the aim of characterizing the changes in gene expression that underlie ESCC progression.

Laser capture microdissection of frozen specimens in collaboration with an experienced pathologist was applied to collect the cells from well-defined tumor areas. Whole human gene expression profiling of ESCC specimens (n=10) and normal esophageal squamous tissue (n=18) was performed using microarray technology. Microarray results were validated by quantitative real-time polymerase chain reaction (qRT-PCR) in a second and independent cohort (n=71) consisting of ESCC (n=25), normal esophagus (n=11), esophageal adenocarcinoma (n=13), gastric adenocarcinoma (n=15) and colorectal cancers (n=7). In order to understand the functional relevance of a newly identified marker gene, siRNA-mediated knockdown was performed in a human esophageal squamous cell carcinoma cell line, KYSE520, and the effects of this treatment were checked by another microarray analysis.

A gene encoding WDR66, WD repeat-containing protein 66, was found whose expression was significantly high in ESCC specimens ($P < 0.0001$). High WDR66 expression was significantly associated with poor overall survival ($P = 0.031$) of patients suffering from ESCC. Multivariate Cox regression analysis revealed that WDR66 expression remained an independent prognostic factor ($P = 0.042$). WDR66 knockdown by RNA interference resulted particularly in changes of the expression of membrane components. This was

validated by qRT-PCR and western blotting experiments in the human squamous cell carcinoma cell line KYSE520, providing independent evidence of the changes of Vimentin (VIM) and occluding (OCLN) expression associated with the WDR66 knockdown. Gene expression level of VIM was significantly down-regulated whereas OCLN expression was significantly higher in cells treated with WDR66 siRNA by qRT-PCR (VIM, $P = 0.0286$; OCLN, $P = 0.0186$). Detection of VIM and OLCN protein by immunoblotting showed that VIM expression was significantly down-regulated while the expression of OCLN was significantly higher in KYSE520 cells treated with WDR66 siRNA. Interestingly, VIM was highly expressed ($P = 0.0008$) while OCLN was less expressed ($P < 0.0001$) in ESCC specimens in comparison to normal esophagus. This may underline a central role of WDR66 for ESCC development. Furthermore, siRNA-mediated knockdown of WDR66 resulted in suppression of cell growth, reduced cancer cell motility and enhanced cancer cell apoptosis after addition of Staurosporine and 5-Fluorouracil.

According to these studies, WDR66 might be a useful biomarker for risk stratification of ESCC. As a positive modulator of epithelial-mesenchymal transition, WDR66 expression is likely to play an important role in ESCC growth and invasion. Moreover, our functional studies point toward an important role of WDR66 for squamous carcinoma cell growth and motility. In the future, WDR66 might become a novel drug target for the treatment of esophageal squamous cell carcinoma.

2. ZUSAMMENFASSUNG

Karzinome der Speiseröhre werden schon lange als besonders gefährlichfür den Patienten und ihreBehandlung wird weiterhin kontrovers diskutiert. Aufgrund der späten Vorstellung von Patienten mit dieser Krankheit und der technischen Schwierigkeit einer adäquaten chirurgischen Resektion bei Vorliegen von erweiterten lokalen und regionalen Rezidiven haben Patienten mit Karzinomen der Speiseröhre eine schlechte Prognose. Das häufigste Karzinom der Speiseröhre ist das Plattenepithelkarzinom des Ösophagus, dessen Verteilung sich über die gesamte Länge der Speiseröhre erstreckt. Um eine maßgeschneiderte Therapie und damit verbesserte Prognose zu erreichen, suchten wir hier nach Genen mit erhöhter Expression in Plattenepithelkarzinomen des Ösophagus. Dazu untersuchten wir die Genexpressionsprofile von Plattenepithelkarzinomen des Ösophagus im Vergleich zu normalem Ösophagus Plattenepithel.

Die Laser-Mikrodissektion von gefrorenem Probenmaterial wurde angewandt, um in Zusammenarbeit mit einem erfahrenen Pathologen (Prof. Michael Vieth, Universität Bayreuth) nur Zellen aus wohldefinierten Tumorbereichen für die Untersuchung zu selektieren. Unter Verwendung der Mikroarray-Technologie wurde ein sogenanntes „Whole human Genome Expression Profiling“ von Ösophagus-Plattenepithelkarzinomproben (n = 10) und gesundem Ösophagus Gewebe (n = 18) durchgeführt. Die Ergebnisse der Microarrayanalyse wurden mittels quantitativer real-time Polymerase-Kettenreaktion (qRT-PCR) in einer zweiten und unabhängigen Kohorte (n = 71), bestehend aus Plattenepithelkarzinomen des Ösophagus (n = 25), gesundem Ösophagus (n = 11), Adenokarzinomen des Ösophagus (n = 13), Adenokarzinomen des Magens (n = 15) und des Kolons (n = 7) validiert. Um die funktionelle Relevanz eines neu identifizierten Markergens zu verstehen, wurde dessen Expression mittels siRNA-vermittelten Knockdown in einer humanen Plattenepithelkarzinom-Zelllinie unterdrückt und die Wirkung dieser Behandlung mit Hilfe einer weiteren Mikroarray-Analyse überprüft.

Ein Gen, das WD repeat Protein enthaltende Gen 66 (WDR66), erwies sich als hochsignifikant überexprimiert in Plattenepithelkarzinomproben ($P < 0,0001$). Eine hohe WDR66 Expression von solchen Karzinomen korrelierte signifikant mit schlechtem Überleben ($P = 0,031$) der Patienten mit Ösophagus-Plattenepithelkarzinomen. Multivariate Cox Regressionsanalyse zeigte, dass die WDR66 Expression ein unabhängiger prognostischer Faktor ($P = 0,042$) blieb. WDR66 Knockdown durch RNA-Interferenz hatte insbesondere Einfluss auf Veränderungen der Expression von Membran-Komponenten. In Plattenepithelkarzinomen des Ösophagus im Vergleich zu gesundem Epithel wurde Vimentin stark exprimiert ($P = 0,0008$), während die Occludin Expression reduziert war ($P < 0,0001$). Die Microarray-Daten wurden mittels qRT-PCR und Western Blot Experimenten validiert. Der siRNA-vermittelte Knockdown von WDR66 führte außerdem zu einer

Unterdrückung des Zellwachstums, zu reduzierter Zellmotilität und einer verstärkten Apoptose der Zellen nach Zugabe von Staurosporin und 5-Fluorouracil.

Die Ergebnisse dieser Studie zeigen, dass WDR66 ein nützlicher Biomarker für die Risikostratifizierung von Patienten mit Ösophagus-Plattenepithelkarzinomen ist. Als positiver Modulator der Epithelial-mesenchymalen Transition (EMT) spielt WDR66 vermutlich eine wichtige Rolle für Wachstum und Invasion von Ösophagus-Plattenepithelkarzinomen. Dafür sprechen auch die Ergebnisse unserer funktionellen Studien im in-vitro System. Darüber hinaus ist WDR66 möglicherweise ein neues Drug-Target für die Behandlung von Plattenepithelkarzinomen des Ösophagus.

3. INTRODUCTION

3.1 ANATOMY OF THE ESOPHAGUS

The esophagus is a muscular canal, measuring 25-30cm in an adult, connecting the throat with the stomach. It lies behind the trachea and the heart, but in front of the spinal column. Just before entering the stomach, the esophagus passes through the diaphragm (Figure 1).

The esophagus can contract and expand to allow food to travel through it and has a peristaltic action, produced by muscles in the esophageal wall, which helps push the bolus through the tube.

The upper esophageal sphincter (UES) is a bundle of muscles at the top of the esophagus. The muscles of the UES are under conscious control, used when breathing, eating, belching, and vomiting. They keep food and secretions from going down the windpipe.

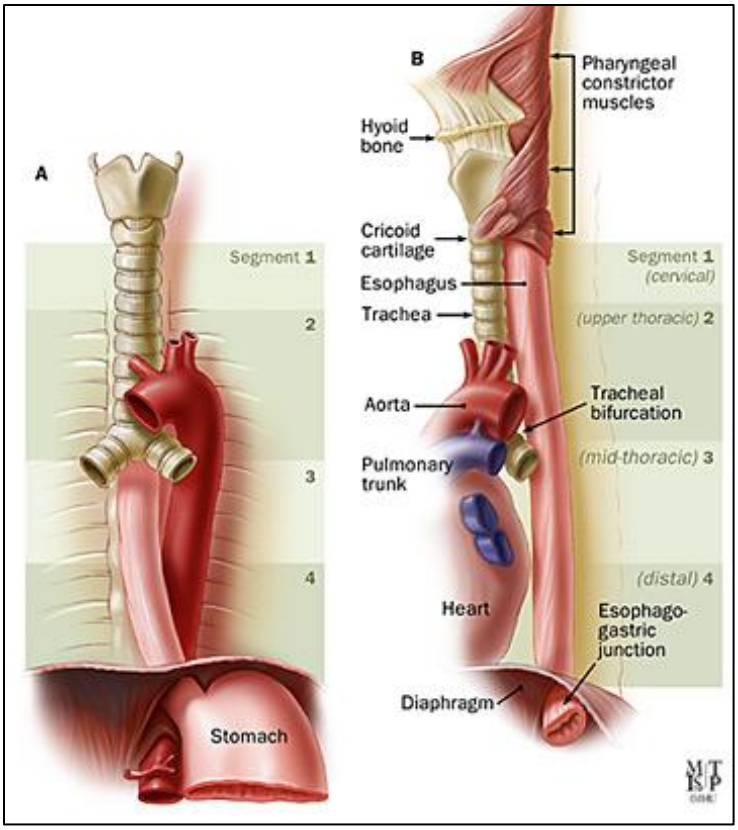

Figure 1. Anatomy of the esophagus (from gi.jhsps.org).

The lower esophageal sphincter (LES) is a bundle of muscles at the low end of the esophagus, where it meets the stomach. When the LES is closed, it prevents acid and stomach contents from traveling backwards from the stomach. The LES muscles are not under voluntary control.

The esophagus itself has several unique properties that distinguish the behavior of cancer in this organ from that of other gastrointestinal malignancies. In contrast to the rest of the gastrointestinal tract, the esophagus has no serosa, thus reducing the resistance against

local spread of invasive cancer cells. Furthermore, the esophagus has an extensive network of lymphatic vessels, allowing for early regional tumor development (Figure. 2).

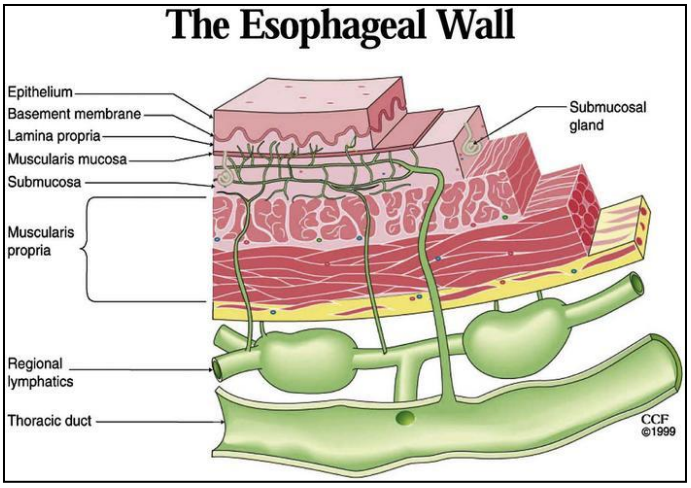

Figure 2. Schematic illustration of the construction of the esophageal wall (from uhhospitals.org).

3.2 ESOPHAGEAL CARCINOMA

Esophageal cancer (or oesophageal cancer) is the eighth most common cancer worldwide, with 481,000 new cases (3.8% of the total) estimated in 2008, and the sixth most common cause of death from cancer with 406,000 deaths (5.4% of the total). These figures encompass both adenocarcinoma and squamous cell carcinoma types. More than 80% of the cases and of the deaths occur in developing countries (Table.1).

Estimated numbers (thousands)	Men			Women			Both sexes		
	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.
World	326	276	335	155	130	146	481	406	481
More developed regions	63	52	85	17	14	21	80	66	106
Less developed regions	262	223	250	137	115	124	399	338	374
WHO Africa region (AFRO)	15	15	15	8	8	8	23	23	23
WHO Americas region (PAHO)	26	23	31	9	7	10	35	30	41
WHO East Mediterranean region (EMRO)	8	8	7	7	6	6	15	14	13
WHO Europe region (EURO)	37	32	38	14	12	13	51	44	51
WHO South-East Asia region (SEARO)	39	36	25	27	24	18	66	60	43
WHO Western Pacific region (WPRO)	197	159	217	88	70	88	285	229	305
IARC membership (22 countries)	88	75	98	37	31	32	125	106	130
United States of America	12	11	16	3	3	4	15	14	20
China	175	143	177	83	67	80	258	210	257
India	28	26	14	19	16	10	47	42	24
European Union (EU-27)	24	21	27	8	7	8	32	28	35

Table 1. Esophageal cancer incidence, mortality and prevalence worldwide in 2008. GLOBOCAN 2008 (IARC), Section of Cancer Information (13/3/2012).

The incidence rates of esophageal cancer vary internationally by more than 15-fold in men (22.3 per 100,000 in Southern Africa compared to 1.4 in Western Africa), and almost 20-fold in women (11.7 per 100,000 in Southern Africa compared to 0.6 in Micronesia/Polynesia). Esophageal cancer is two to four times more common among men than women (Figure.3). The highest mortality rates are found in both sexes in Eastern and Southern Africa, and in Eastern Asia [1].

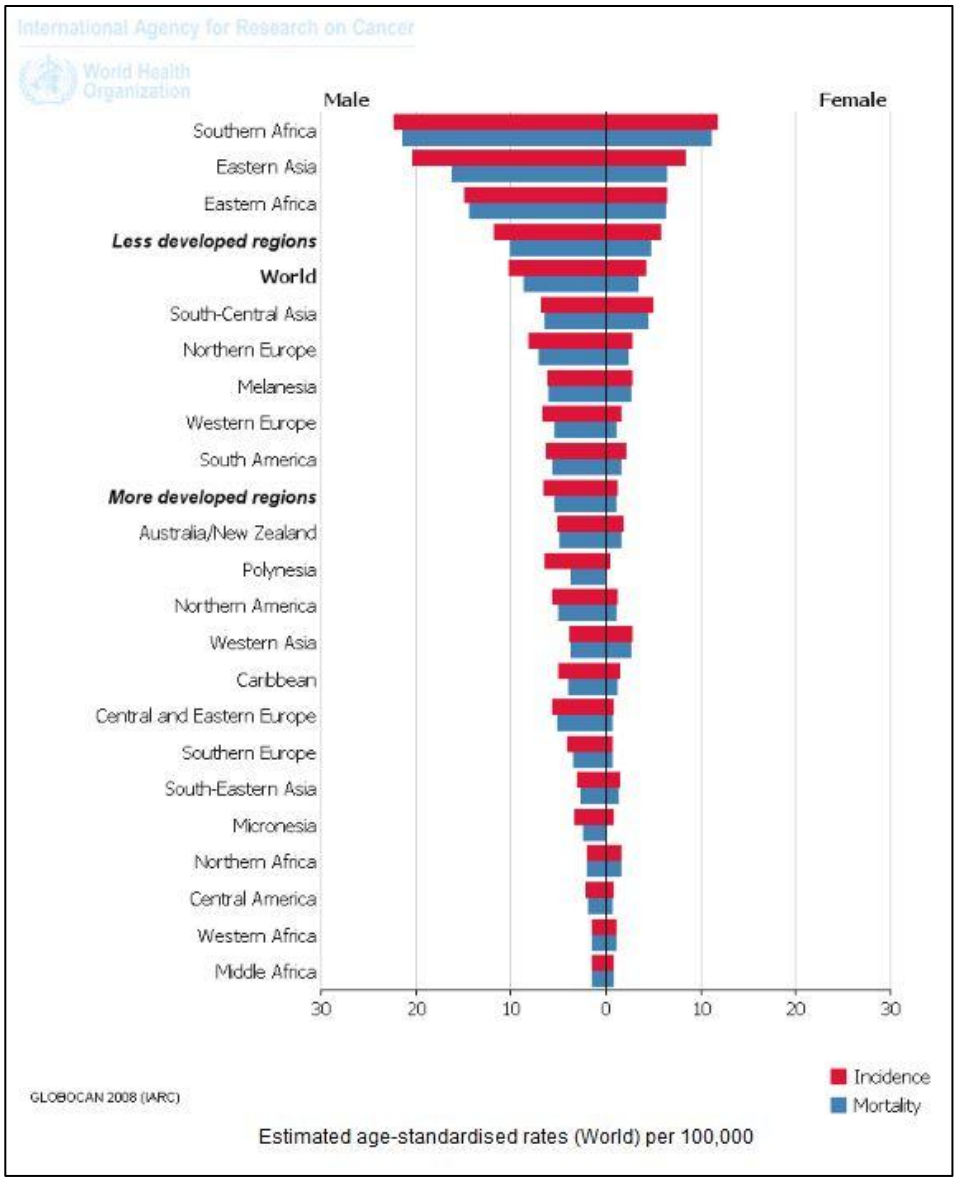

Figure 3. Esophageal cancer incidence and mortality worldwide in 2008. Estimated age-standardised rates (world) per 100,000. Figure was generated using the Globocan 2008 website (globocan.iarc.fr). The estimates are based on the most recent data available at the International Agency for Research on Cancer (IARC)

3.3 LIMITATION OF CURRENT DETECTION METHODS FOR ESOPHAGEAL CANCER

Survival and prognosis of esophageal cancer patients depend mainly on the stage of the tumor at the time of detection. At present, gastrointestinal endoscopy remains the primary screening tool by which the suspected lesions can be biopsied for histopathological analysis. This invasive test, even though it has been proved to increase the detection of early tumor and therefore can prolong the survival of the patient, is generally considered to be inconvenient and painful [2]. Because of its limitation, there is an urgent need for discovery of novel predictive markers for esophageal cancer.

3.4 MICROARRAY-BASED GENE EXPRESSION ANALYSIS

3.4.1 PRINCIPLES OF MICROARRAY-BASED GENE EXPRESSION ANALYSIS

Microarray technology is a powerful application to measure genome-wide changes in mRNA expression levels from various samples [3]. The whole human genome microarray from Agilent containing 44,000 60-mer oligonucleotides represents approximately 41,000 human genes and transcripts based on the most current genomics information available. Using gene expression, researches are trying to find out which genes are expressed ubiquitously in all human tissues, and which are expressed in a tissue-specific manner, e.g. only in tumor tissues.

3.4.2 SAMPLE PREPARATION, TECHNICAL ISSUES, DATA ANALYSIS

Transcriptional profiling with the use of microarray analysis or other techniques is sensitive to the manner in which the samples are collected and handled. Even the surgical manipulation can influence what we observe as gene-expression patterns. This becomes clearer when we imagine what happens to the tumor during surgery. One of the first steps

during surgical removal of malignant tumor is the ligation of the arterial blood supply of the tumor. From this moment on, hypoxemia of the tumor at body temperature occurs. The duration between ligation and final removal of the tumor can vary considerably, and, under clinical conditions, may not be reduced to a constant interval. After removal of the tumor, logistical constraints may lead to further considerable delay before the tumor is finally transferred to –80°C. Therefore, this lengthy process could lead to a considerable extent of RNA-degradation.

After successful sampling of the tumor specimen, biopsy or blood sample, a complex process of preparing high quality RNA or cDNA for hybridization on the microarray has to be performed (Figure 4). The first step is the cryo-cutting of the frozen sample material to prepare several sections, which can be used for further processing and in parallel for histological analysis of the sample material by an experienced pathologist. Histological analysis is mandatory to ascertain that the sample is representative. Furthermore, samples with a high amount of necrotic areas, fatty tissue and contaminating cells should be excluded to reduce signal noise.

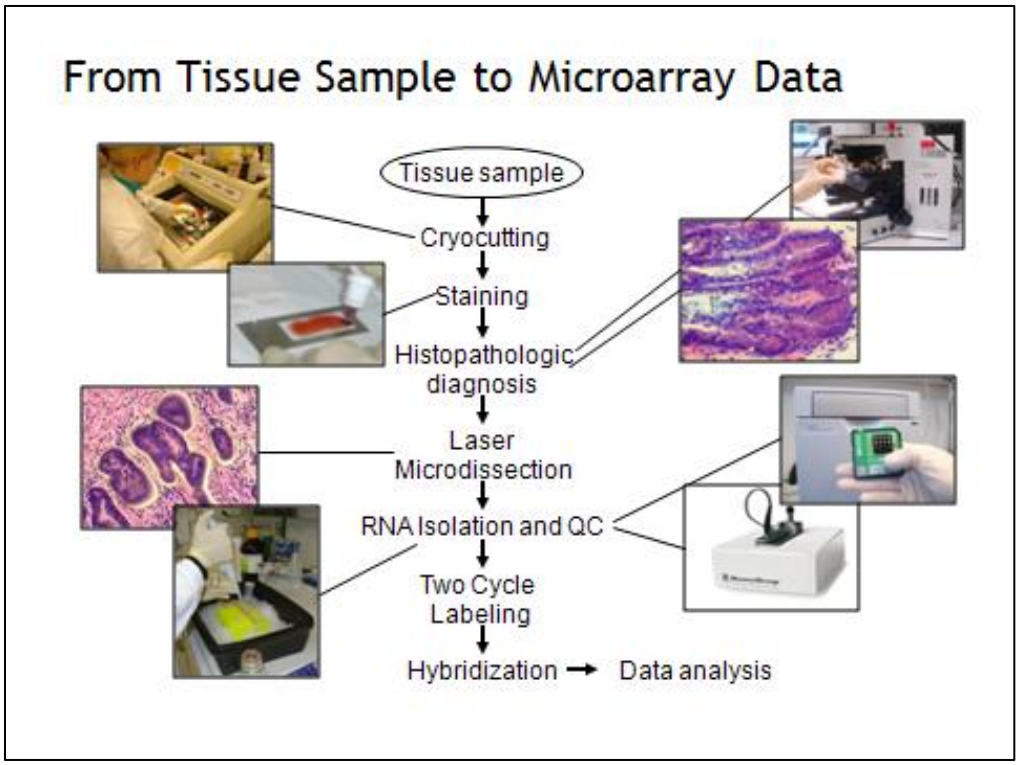

Figure 4. From tissue samples to microarray data (kindly supplied by Dr. Kemmner).

A further improvement could be laser microdissection (LMD) of the tissue of interest. Because of the complex process of sample preparation and hybridization during microarray, analysis artifacts are generated which lead to a high signal to noise ratio. Therefore one should try to work with sample material which is as pure as possible. Of course, the process of exclusion of everything other than e.g. pure tumor tissue sharpens the analysis. A disadvantage is that molecular processes or alterations within the surrounding stroma cannot be detected any more. The last steps include RNA-extraction, RNA quality and quantity assessment, and labeling of the sample sequence. Depending on how much sample RNA is available, amplification procedures have to be included (Figure 5).

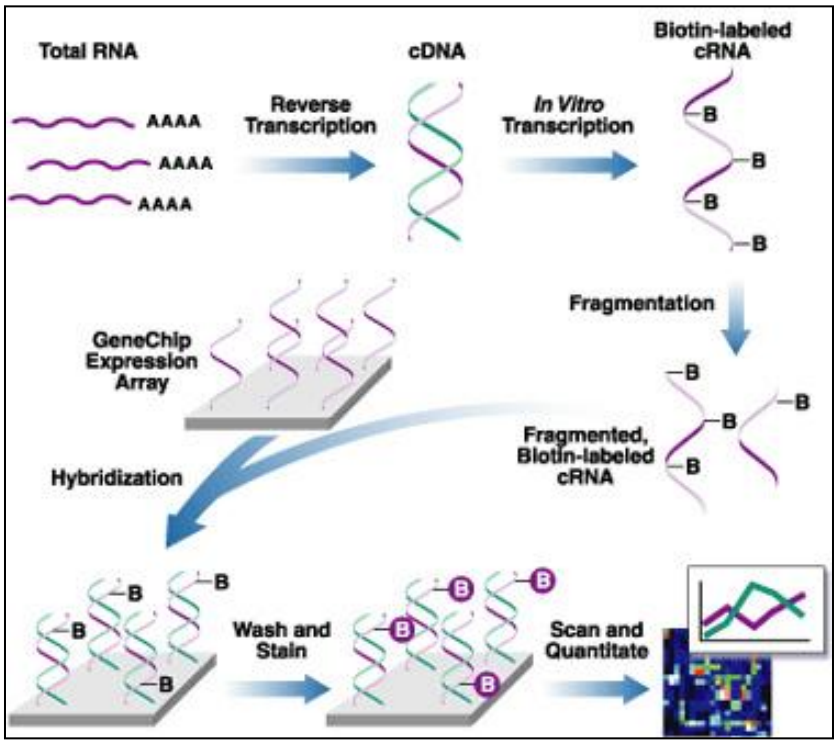

Figure 5. Schematic overview on the different steps while performing an Affymetrix GeneChip experiment (from dkfz.de).

Finally, the labeled sample sequence is hybridized on the microarray. The array is then washed to remove any RNA that is not stuck to microarray and then stained with the fluorescent molecule. Lastly, the entire array is scanned with a laser and the information is kept in a computer for quantitative analysis of what genes were expressed and at what approximate level.

A major challenge of DNA microarray technology is of course the massive data output. Several approaches are used to classify patients on the basis of expression profiles: Fisher's linear discriminant analysis, nearest-centroid prediction rule, and support vector machines, among others [4,5]. Recently, a critical review summarized some of the statistical flaws commonly found in published microarray studies [5].

To estimate the accuracy of a classification method, the standard strategy is via a training–validation approach, in which a training set is used to identify the molecular signature and a validation set is used to estimate the proportion of misclassifications [6]. One approach is to randomly split the initial dataset into two subsets. One part, the so called training set, is used for identifying the gene signature which can be used for discrimination among the tumor subtypes or between tumor and normal tissue. The other part of the cohort, the so called test set, is then used to evaluate the classifier developed from the training set. The fundamental principle is that the samples used for validation must not have been used in any way before being tested. Most importantly, the outcome information of the tested samples must not have been used for identification of the gene signature which leads to the classifier.

3.4.3 ADVANTAGES OF MICROARRAY-BASED GENE EXPRESSION ANALYSIS

Figure 6 gives a brief overview of the questions which might be answered with the help of microarray-based gene expression analysis. Gene expression can analyze predisposal testing for hereditary diseases; enable an accurate diagnosis of unknown carcinomas; decipher genes with prognostic relevance for survival or metastasis formation; find the responders to hormonal or chemotherapy, and detect marker gene expression in non-invasive assays.

Regulated genes are categorized in terms of what they are, important relationships between other genes may emerge. Observing these interactions could be helpful to explain more possible associations among the genes of interest, as well as to identify underlying

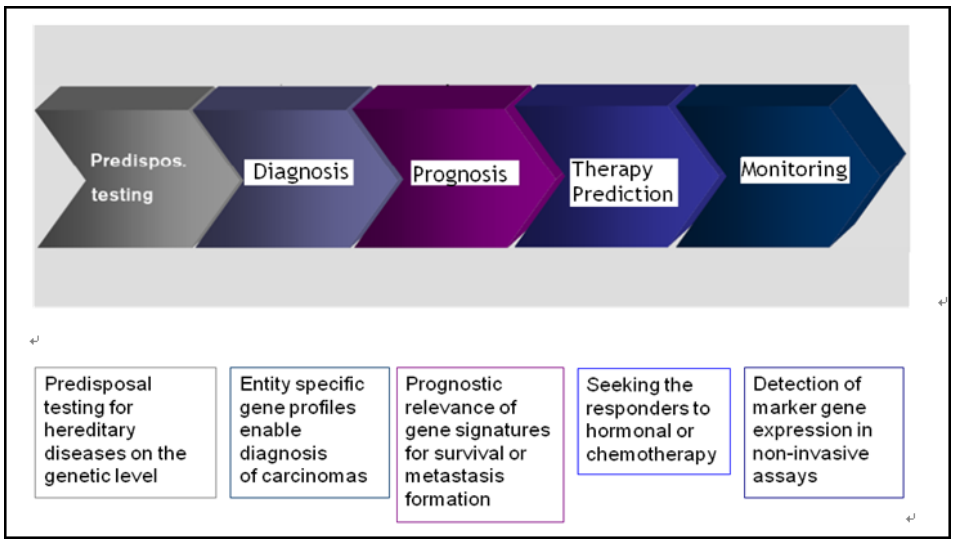

Figure 6. Microarray applications in cancer research (kindly supplied by Dr. Kemmner).

biological processes or pathways. Therefore gene expression profiling is necessary to simultaneously monitor and study the effects of a given treatment on gene expression.

Microarray technology produces reliable expression profiles. Using this information, one can generate new hypotheses about biology or test existing ones. However, the size and complexity of these experiments often results in a wide variety of possible interpretations. In many cases, analyzing expression profiling results takes far more effort than performing the initial experiments [7].

3.5 TAQMAN QUANTITATIVE RT-PCR

TaqMan quantitative RT-PCR was first reported in 1991 by researchers at Cetus Corporation and the technology was subsequently developed by Roche Molecular Diagnostics for diagnostic assays and by Applied Biosystems for research applications. It has the ability to monitor the progress of the PCR as it occurs. Reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. In one-step qRT-PCR, total RNA is directly used and cDNA synthesis is followed by PCR cycling without additional handling.

TaqMan uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles (Figure.7). TaqMan probes are dual labeled hydrolysis probes. They utilize the 5' exonuclease activity of the enzyme Taq polymerase for measuring the amount of target sequences in the samples. The probes normally consist of an 18-22 bp oligonucleotide probe which is labeled with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end. Briefly speaking, the method is based on the 5'-3' exonuclease activity of the Taq DNA polymerase, which results in cleavage of fluorescent dye-labeled probes during PCR. The intensity of fluorescence is then measured by a Sequence Detection System (SDS 2.2).

Specific hybridization between probe and target is required to generate fluorescent signal, and the Taqman probes can be labeled with different, distinguishable reporter dyes, which allows amplification of two distinct sequences in one reaction tube. Additionally, data processing is easier analyzed and accurately quantified by software.

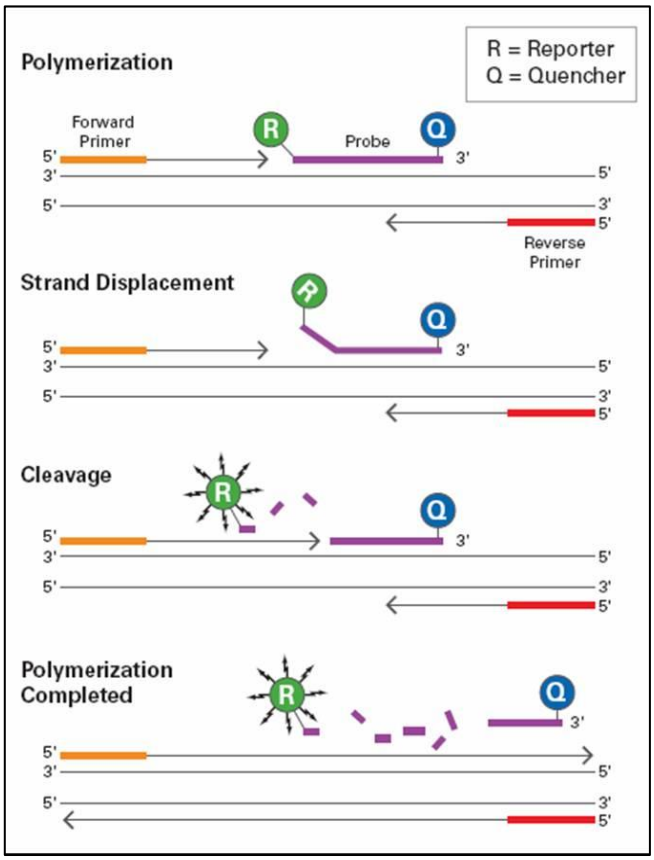

Figure 7. Workflow of Real-time detection via TaqMan primer & probe technology (from http://technologyinscience.blogspot.de/).

The standard curve method for absolute quantitation was used here (Figure.8). Amplification of an endogenous control may be performed to standardize the amount of sample RNA added to a reaction. To quantify the WDR66 gene expression, a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control.

$$\Delta Ct = Ct_{WDR66} - Ct_{GAPDH}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{wildtype}$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Figure 8. Calculation of delta-Ct (Cycle Threshold), delta-delta-Ct, and finally fold change of target gene expression.

3.6 GENE KNOCKDOWN (SIRNA)

Gene knockdown refers to techniques by which the expression of one or more of an organism's genes is reduced, either through genetic modification (a change in the DNA of one of the organism's chromosomes) or by treatment with a reagent such as a short DNA or RNA oligonucleotide with a sequence complementary to either an mRNA transcript or a gene. If genetic modification of DNA is done, the result is a "knockdown organism". If the change in gene expression is caused by an oligonucleotide binding to an mRNA or temporarily binding to a gene, this results in a temporary change in gene expression without modification of the chromosomal DNA and is referred to as a "transient knockdown".

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 nucleotides in length, that play a variety of roles in biology. The most notable role of siRNA is its involvement in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene (Figure.9).

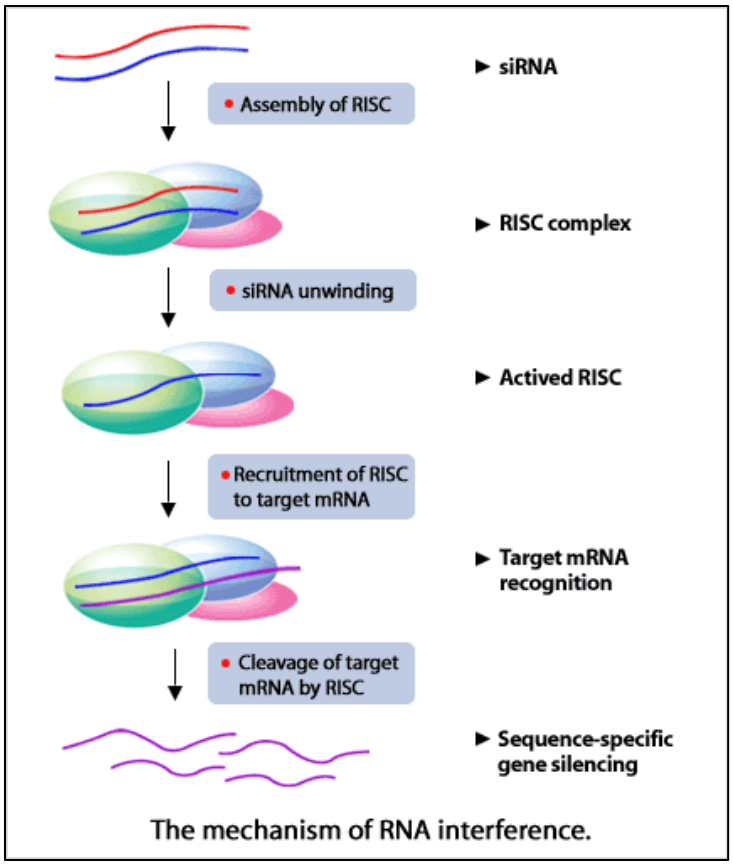

Figure 9. Mechanism of small interfering RNA (siRNA) silencing (from us.bioneer.com).

Given the ability to knock down, in essence, any gene of interest, RNAi via siRNAs has generated a great deal of interest in both basic and applied biology. There are an increasing number of large-scale RNAi screens that are designed to identify the important genes in various biological pathways. Because disease processes also depend on the activity of multiple genes, it is expected that in some situations turning off the activity of a gene with a siRNA could produce a therapeutic benefit.

3.7 WDR GENE FAMILY

WD-repeat protein family is a large family found in all eukaryotes and is implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control, autophagy and apoptosis [8]. According to whole-genome sequence analysis,

there are 136 WD-repeat proteins in humans which belong to the same structural class [9]. The known functions of the proteins that possess WD-repeats span a broad spectrum, from signal transduction to cell cycle control [10,11].

In humans, the WDR66 (WD repeat-containing protein 66) gene is located on chromosome 12 (12q24.31) and contains 23 exons. The coding cDNA contains 3768 nucleotides and encodes a protein of 1149 amino acids. The WD repeat-containing protein 66 contains 9 highly conserved WD40 repeat motifs and an EF-hand-like domain. A genome-wide association study identified a single-nucleotide polymorphism located within intron 3 of WDR66 associated with mean platelet volume [12]. These repeating units are believed to serve as a scaffold for multiple protein interactions with various proteins [13].

3.8 AIM: WDR66 FUNCTIONAL ANALYSIS

Esophageal squamous cell carcinoma is one of the most lethal malignancies of the digestive tract and in most cases the initial diagnosis is established only once the malignancy is in the advanced stage [14]. Poor survival is due to the fact that esophageal squamous cell carcinoma frequently metastasizes to regional and distant lymph nodes, even at initial diagnosis. Treatment of cancer using molecular targets has brought promising results and attracts more and more attention [15-18]. In order to achieve a more tailored therapy and, consequently, improved prognosis, we studied gene expression profiles of esophageal squamous cell carcinoma in comparison to that of normal esophageal squamous epithelium with the aim of characterizing the changes in gene expression that underlie esophageal squamous cell carcinoma progression.

Here, we try to assess whether one newly identified candidate molecule, WD repeat-containing protein 66 (WDR66), located on chromosome 12 (12q24.31), might be a useful biomarker for risk stratification

Another aim was to investigate the functional role of WDR66 in ESCC. Therefore, an in vitro siRNA-based knockdown of WDR66 in esophageal squamous cell carcinoma cell

lines was established, followed by functional assays for cell growth, cancer cell motility and cancer cell apoptosis.

4. MATERIAL

4.1 TISSUE SPECIMENS

Gene expression profiling using whole-human-genome microarrays (Affymetrix U133plus2) was performed on 10 esophageal squamous cell carcinoma and 18 normal esophageal biopsies collected from 28 individuals. Normal healthy esophageal biopsies were collected from patients with esophageal pain but diagnosed as normal squamous without pathological changes. Surgical specimens of chemotherapy-naive patients with known ESCC of histological grading G1, UICC stages II and III, who had undergone esophagectomy, were obtained from the tumor bank of Charité Comprehensive Cancer Center. Patients' age ranged from 22 to 83 years, with a median age of 59 years.

Real time quantitative PCR validation was performed on the validation cohort consisting of 71 specimens (patients), including esophageal squamous cell carcinoma (n=25), normal esophagus (n=11), esophageal adenocarcinoma (n=13), gastric adenocarcinoma (n=15) and colorectal cancers (n=7). Patients' age ranged from 24 to 79 years, with a median age of 63 years. All samples were snap-frozen in liquid nitrogen and stored at -80°C.

Moreover, 15 formalin-fixed, paraffin-embedded human esophageal tissue biopsies with equal representation of normal and esophageal squamous cell carcinoma were obtained for in situ hybridization.

All samples were snap-frozen in liquid nitrogen and stored at -80°C. We obtained tissue specimens from all subjects with informed written consent (approved by the local ethics committee of the Charité-Universitätsmedizin, Berlin). Each single specimen included in this study was histopathologically approved according to grade and stage by an experienced pathologist (MV, University Bayreuth).

4.2 CELL CULTURE

4.2.1 CELL LINES

KYSE520 (Human Japanese esophagus carcinoma squamous epithelial cell line), obtained from the American Type Culture Collection (ATCC, Manassas, USA), was used in the beginning to test which has the high expression of WDR66 gene.

Cell pellets of OE33, SW480, HT29, HCT116, LS174T, Caco2, HL60, HEK293, Daudi, CapanI, MCF7, MDA-MB231 and MDA-MB435 were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and processed for RNA preparation.

4.2.2 MEDIUM AND OTHER COMPONENTS

RPMI1640 Roswell Park Memorial Institute Medium (PAA Laboratories GmbH, Pasching, AUT)

PBS Phosphate buffered saline, without Ca/Mg, 1x (PAA Laboratories GmbH, Pasching, AUT)

FCS Fetal calf serum, 1x (Hyclone, Logan, USA)

P/S Penicillin/Streptomycin, 100x (PAA Laboratories GmbH, Pasching, AUT)

L-Glutamin, 200mM (PAA Laboratories GmbH, Pasching, AUT)

Gentamincin, 2x (PAA Laboratories GmbH, Pasching, AUT)

T/E Trypsin/Ethylenediaminetetraacetic Acid 1x (PAA Laboratories GmbH, Pasching, AUT)

4.3 LASER CAPTURE MICRODISSECTION

Cellcut (MMI AG, Eching, GER)

Nikon TE300 microscope (Nikon Corp., Tokyo, JPN)

4.4 siRNA Transfection

DharmaFECT Transfection Reagents Kit (Thermo Fisher Scientific, Massachusetts, USA)

siRNA sense 5' – **GuuACuAAAGGuGAGCAuA** - 3' corresponding to WDR66 mRNA was chemically synthesized by Sigma-Proligo (Munich, GER).

4.5 RNA isolation

RNeasy Mini Kit (Qiagen Inc., Valencia, USA)

QIAshredder Kit (Qiagen Inc., Valencia, USA)

DEPC water (Invitrogen GmbH, Karlsruhe, GER)

4.6 RNA quantity and quality check

NanoDrop ND-1000 (PEQLAB Biotechnologie GmbH, Erlangen, GER)

2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA)

RNA 6000 Nano Kit (Agilent Technologies Inc., Santa Clara, USA)

RNA Nano Chip (Agilent Technologies Inc., Santa Clara, USA)

4.7 TaqMan quantitative RT-PCR

TaqMan Gene Expression Assay-on-Demand (Applied Biosystems, Life Technologies Corporation, Carlsbad, USA)

RNA UltraSense™ One-Step quantitative RT-PCR Kit (Invitrogen GmbH, Karlsruhe, GER)

ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, Carlsbad, USA)

MicroAmp™ Optical 384-well Reaction Plate (Applied Biosystems, Life Technologies Corporation, Carlsbad, USA)

4.8 AFFYMETRIX MICROARRAY-BASED GENE EXPRESSION ANALYSIS

GeneChip Hybridization, Wash and Stain Kit (Affymetrix Inc., Santa Clara, USA)

Affymetrix Human Genome U133plus 2.0 array (Affymetrix Inc., Santa Clara, USA)

Gene Chip Operation Software (Affymetrix Inc., Santa Clara, USA)

GeneSpring GX10.2 (Agilent Technologies Inc., Santa Clara, USA)

DNA Microarray Hybridization Oven (Agilent Technologies Inc., Santa Clara, USA)

Microarray G2565BA fluorescent scanner (Agilent Technologies Inc., Santa Clara, USA)

4.9 IN SITU HYBRIDIZATION

DIG/RNA labelling kit (Böhringer, Mannheim, GER)

4.10 WESTERN BLOTTING ANALYSIS

polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA).

Mouse monoclonal anti-human vimentin antibody, V5255, 1:200, approximately 54kDa (Sigma Aldrich Corporation, St. Louis, USA).

Rabbit polyclonal anti-human occludin antibody, 71-1500, 1:500, at 65kDa (Invitrogen GmbH, Karlsruhe, GER).

Beta actin antibody, 1:2000, ab8226 (Abcam plc, Boston, USA)

Sodium dodecyl sulfate (Carl Roth GmbH + Co. KG, Karlsruhe, GER)

ECL kit (Amersham Pharmacia Biotech, Piscataway, USA).

FUJIFILM LAS1000 Luminescent Image Analyzer (FUJIFILM Holdings Corporation, Tokyo, JPN)

4.11 CELL MOTILITY ASSAY

Transwell® Permeable Support Inserts Coated with Cultrex® Basement Membrane Extract (Corning Costar, Lowell, USA)

4.12 WOUND HEALING ASSAY

CytoSelect™ 24-Well Wound Healing Assay (Cell Biolabs Inc. San Diego, USA)

4.13 APOPTOSIS ANALYSIS

POLARstar Omega (BMG Labtech, Offenburg, GER)

Calcein (Sigma Aldrich Corporation, St. Louis, USA)

Staurosporine (friendly supplied by Charité, Berlin, GER)

5-Fluorouracil (kindly supplied by Charité, Berlin, GER)

5. METHODS

5.1 CELL CULTURE

KYSE520 was cultured in RPMI medium containing 10% FBS GOLD, 1% penicillin/streptomycin and 2mM glutamine at 37°C in a humidified and 5% CO_2-containing atmosphere. FBS GOLD is a chromatographically purified and fractionated serum that is recombined in a defined composition, which prevents batch-to-batch variations. Morphology was observed and recorded every day under the light microscope. Growth curve assay was performed in the beginning and during the mid-term of cell culture.

Subculturing with medium renewal was done every 2 to 3 days. Therefore, the culture medium was removed from the adherent cells and washed once with PBS (containing 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 mg/ml gentamycin) to remove all traces of serums which could inhibit the protease and collagenase in the next step. The surface area of a 75 cm^2 flask was covered with trypsin, which contains protease and collagenase activities in an isotonic, phosphate buffer solution with EDTA. The flask was incubated for 10 minutes at 37°C. When all cells had detached, the trypsin buffer had to be removed. Then complete culture medium was added, and cells were counted in an improved Neubauer counting chamber and diluted (for seeding in 6- 24 well-plates) or passaged (in a new T75 flask in a ratio of 1:5 or 1:10) as needed.

5.2 SIRNA TRANSFECTION

Transfection was carried out in 6-well plates with DharmaFECT lipid transfection. A cell density of $1x10^5$ cells/well at a minimum is needed to isolate RNA for the downstream experiments. For preparation of siRNA-based gene knockdown, the cells were detached, counted, diluted in antibiotic-free complete medium, seeded in 6-well plates and incubated for 24 hours at 37°C with 5% CO_2. Prepare the transfection reagents siRNA 50ng per well. Control siRNA (AllStar) and mock transfection (NTC) were included as negative controls. The medium from the wells of the 6-well plates was removed, once washed with PBS and

2 ml of the transfection medium was added. The assay was incubated for 48 hours without medium replacement.

5.3 RNA ISOLATION

Trypsinized and collected cells were washed twice with 1x PBS at 300 x *g* for 5 minutes. Put 350µl RLT buffer (plus 1% beta -Mercaptoethanol) to the cell pellet, mix well and transfer to QIAshredder spin column, centrifuge for 2minutes at 15000rpm, then discard the filter and add 350µl cooled 70% ethanol to the homogenized lysate, mix well and transfer to the RNeasy spin column. Centrifuge the RNeasy spin column at 12000rpm for 15 seconds, discard the waste in the collecting tube, then add 350µl RW1 buffer, once again centrifuge at 12000rpm for 15 seconds. To eliminate genomic DNA contamination, mix 70µl RDD buffer plus 10µl DNase for each sample, carefully add to the column, and incubate at room temperature for 15 minutes. Wash with 350µl RW1 buffer, centrifuge at 12000rpm for 15 seconds. Add 500µl RPE buffer, centrifuge at 12000rpm for 15 seconds, and discard the waste. Wash again with 80% ethanol and centrifuge at 12000rpm for 2 minutes. Discard the waste and centrifuge again at 15000rpm for 5minutes. Change to the new collecting tube, and carefully add 14µl RNeasy-free water to the center of the spin column membrane, then centrifuge at 15000rpm for 1 minute. The RNA was kept at -80 °C until the microarray experiment was started.

5.4 RNA QUANTITY AND QUALITY CHECK

The extracted RNA concentration was measured by ND-1000 spectrophotometer (NanoDrop). RNA quantity is measured at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity. A ratio of 2 is accepted as pure. If the ratio is lower than 2, it may indicate the presence of phenol, protein or other contaminants. The 260/230 ratio is used as a second measure of nucleic acid purity. If this ratio is lower than 2, it may indicate the presences of contaminants which absorb at 230 nm, like EDTA or carbohydrates. RNA quality was checked using the Bioanalyzer with the Agilent RNA 6000

Kit and carried out according to the manufacturer's protocol. The Bioanalyzer uses capillary electrophoresis to determine the RIN (RNA Integrity Number) value. The calculation of the RIN value is based on the area under the peaks of the 28S and the 18S rRNA. A 2:1 ratio indicates intact total RNA and gives a RIN value of 10, a RIN value of 1 in contrast indicates degradation of RNA. For further downstream experiments, RNA samples with RIN values less than 8 were excluded. (Figure 10)

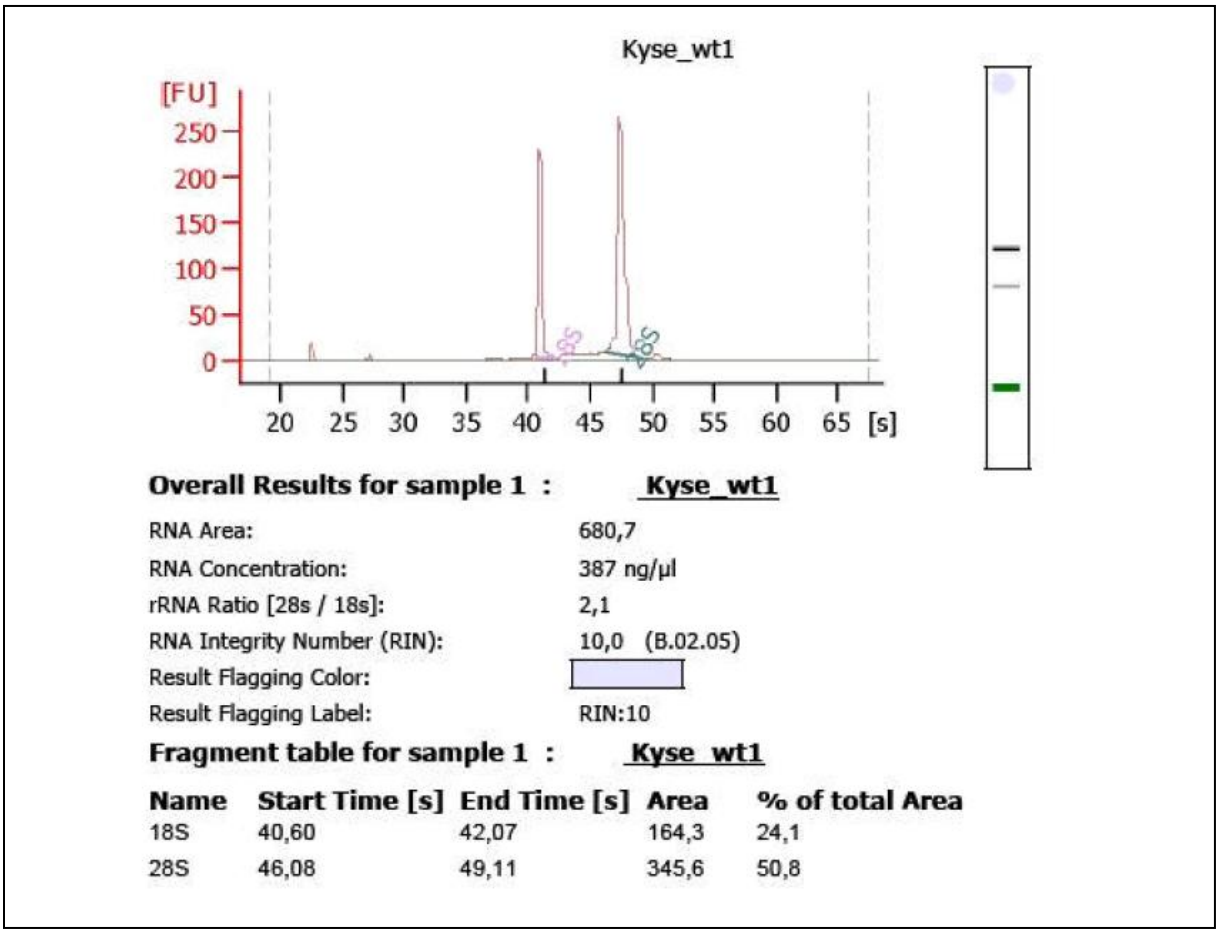

Figure 10. Result example for an Electrophoretic Nano Assay performed on Bioanalyzer, which indicates RNA integrity number (maximal RIN=10), and other parameters e.g. RNA concentration.

5.5 KNOCK-DOWN EFFICIENCY

The knockdown efficiency was determined using real time PCR as described in Chapter 3.6. The RNA UltraSense™ One-Step Quantitative RT-PCR system in combination with TaqMan® Gene Expression Assays was used because of the little amount of RNA.

Based on the previous study from our group, the knock-down experiment succeeded within the first several tries. The transfections repeated three times were successful (Figure 11), showing that the condition of this transfection was feasible for cell lines KYSE520.

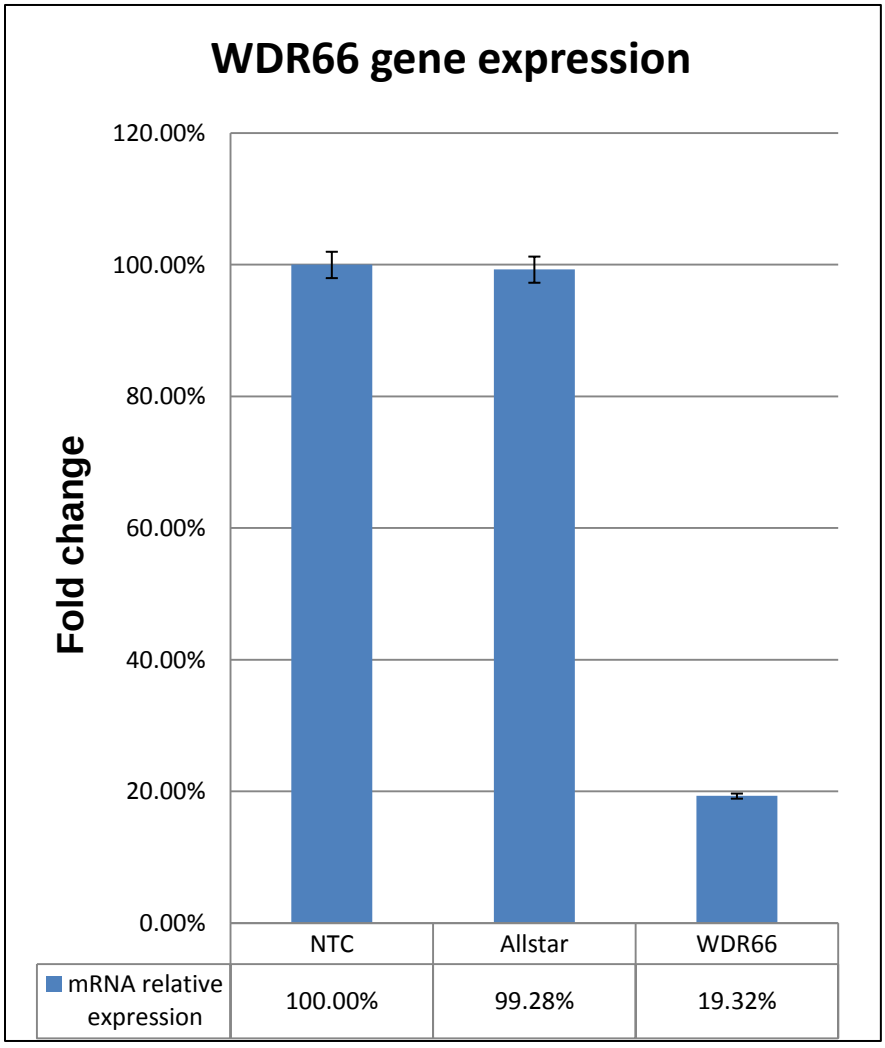

Figure 11. Knock-down efficiency of KYSE520 cell lines. Error bar show the standard deviation from three repetitions.

The WDR66 gene expression and knock-down quantification by realtime PCR was also performed to ensure that during the in vivo experiment the knock-down efficiency was still

maintained. From the figures above (Figure 12), it is clearly apparent that, 4 days after the siRNA transfection, KYSE520 kept good knock-down efficiency.

	day0	day1	day2	day3	day4
NTC	100%	100%	100%	100%	100%
Allstar	110%	82%	98%	99%	108%
WDR66	14%	32%	34%	38%	41%

Figure 12. Fold change of WDR66 expression in knocked-down KYSE520 cells after day1, day2, day3 and day4 of transfection.

5.6 TAQMAN QUANTITATIVE RT-PCR

Pipette all the reaction reagents and isolated sample RNA to a thermal cycling 384-well plate as shown in Table 2, making a triplet for each sample, and then perform the assay on Applied Biosystems 7900HT. Using the standard curve and absolute quantification method, and set the program to 50°C for 15 minutes (cDNA synthesis), 95°C for 2 minutes, then repeat the following steps 50 times: 95°C for 15 seconds and 60°C for 30 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen to be the endogenous control for calculating delta-Ct (cycle threshold) later by analyzing the data.

Components	Vol. per reaction	Vol. per triplet
RNA Ultrasense™ Reaction Mix **5x**	**2.0** µl	**7.00** µl
Expression Assay-on-Demand GAPDH **20x**	**0.5** µl	**1.75** µl
Expression Assay-on-Demand WDR66 **20x**	**0.5** µl	**1.75** µl
RNA Ultrasense™ Enzyme Mix	**0.5** µl	**1.75** µl
ROX Reference dye	**0.2** µl	**0.70** µl
DEPC water	**5.8** µl	**20.30** µl
Template (total RNA)	**0.5** µl	**1.75** µl
Total volume	**10.0** µl	**35.00** µl

Table 2. RNA UltrasenseTM one-step qRT-PCR system components.

5.7 AFFYMETRIX MICROARRAY-BASED GENE EXPRESSION ANALYSIS

5.7.1 cDNA SYNTHESIS FROM TOTAL RNA

The quantified and qualified RNA (10 ng) was used for synthesizing cDNA. The following protocol is designed for 10 ng RNA.

a) Prepare the poly-A RNA dilutions

Add 2µl of the Poly-A control stock solution to 38µl of control dilution buffer to prepare the first dilution (1:20). Mix thoroughly and spin down quickly to collect all of the liquid at the bottom of the tube. Then make the second dilution (1:50), third dilution (1:50) and fourth dilution (1:10, finally 500,000-fold dilution). Mix thoroughly on a vortex mixer and spin down briefly.

b) cDNA synthesis, first-cycle

Add the Poly-A Controls Mix to a dark microcentrifuge tube, mix briefly. Denature the primer and the template by incubating the reaction at 70°C in a circulating water bath for 6 minutes. Then place the reactions on ice and incubate for 5 minutes.

Components	Volume
Diluted RNA	2 µl
RNase-free Water	16 µl
T7-Oligo(dT) Primer, 50 µM	2 µl
Total Volume	**20 µl**

Table 3. Preparation of Total RNA Sample/T7-Oligo(dT) Primer/Poly-A Controls Mix.

Immediately prior to use, gently mix First-Cycle, First-Strand Master Mix listed below and put on ice. Add 5µl of First-Cycle, First-Strand Master Mix to each sample tube and mix by pipetting up and down. Incubate samples at 40°C in a circulating water bath for 1 hour. Move samples to a 70°C circulating water bath and incubate for 10 minutes to deactivate RT enzyme. Then put samples on ice and incubate for 5 minutes.

Components	Volume
5× First Strand Reaction Mix	2.0 µl
0.1 M DTT	1.0 µl
10mM dNTPs	0.5 µl
SuperScript II	1.0 µl
RNase Inhibitor	0.5 µl
Total Volume	**5.0 µl**

Table 4. Preparation of First-Cycle, First-Strand Master Mix.

Prepare sufficient First-Cycle,Second-Strand Master Mix below, add to each sample for a total volume of 20µl. Incubate for 2 hours at 16°C, then 10 minutes at 75°C. Then put samples on ice and incubate for 5 minutes.

Components	Volume
RNase-free Water	4.8 µl
Freshly diluted $MgCl_2$, 17.5mM	4.0 µl
10mM dNTPs	0.4 µl
E.coli DNA Polymerase I	0.6 µl
RNase H	0.2 µl
Total Volume	**10.0 µl**

Table 5. Preparation of First-Cycle, Second-Strand Master Mix.

Prepare sufficient First-Cycle, IVT Master Mix for all of the samples. Transfer 30 µL of First-Cycle, IVT Master Mix to each cDNA sample. Incubate for 16 hours at 37°C.

Components	Volume
10X Reaction Buffer	5 µl
ATP Solution	5 µl
CTP Solution	5 µl
UTP Solution	5 µl
GTP Solution	5 µl
Enzyme Mix	5 µl
Total Volume	**30 µl**

Table 6. Preparation of First-Cycle, IVT Master Mix.

c) Cleanup the cRNA, first-cycle

Add 50µl of RNase-free water to the cRNA sample, and then add 350µl of IVT cRNA Binding Buffer and mix well by pipetting. Add 250µl 100% ethanol and mix thoroughly by pipetting. Transfer the 700µl of the cRNA sample to an RNeasy Cleanup Spin Column in a 2ml collection tube. Centrifuge the sample at 4°C for 30 seconds at 13000 rpm. Transfer the RNeasy column to a new collection tube and add 500µl of IVT cRNA Wash Buffer to the column. Centrifuge the sample at 4°C for 30 seconds at 13000rpm. Discard the flowthrough. Re-use the collection tube. Pipet 500µl 80% ethanol to the column. Centrifuge the sample at 4°C for 60 seconds at 13000rpm. Open the cap of the spin column and centrifuge for 5 minutes at 13000rpm. Discard flow-through and Collection Tube. Elute the cleaned cRNA sample by transferring the RNeasy column to a new 1.5ml dark collection tube. Add 13µl RNase-free water directly onto the RNeasy filter membrane. Put sample for 60 seconds on ice, centrifuge at 4°C for 1 minute at 13000rpm. Then put the purified RNA on ice.

d) cDNA synthesis, second-cycle

Make a fresh dilution of the Random Primers to cleanuped cRNA. Incubate for 10 minutes at 70°C in a circulating water bath for 10 minutes. Then Place the reactions on ice and incubate for 5 minutes.

Mix Second-Cycle, First-Strand Master Mix listed below. Add 9µl of Second-Cycle, First-Strand Master Mix to each sample tube and mix by pipetting up and down. Incubate samples at 42°C in a circulating water bath for 1 hour. Then move samples to ice and incubate for 5 minutes. Add 1 µl of RNase H to each sample and incubate for 20 minutes at

37°C. Heat the sample at 95°C for 5 minutes, and then put samples on ice and incubate for 5 minutes.

Components	Volume
5× First Strand Reaction Mix	4 µl
0.1 M DTT	2 µl
10mM dNTPs	1 µl
SuperScript II	1 µl
RNase Inhibitor	1 µl
Total Volume	**9 µl**

Table 7. Preparation of Second-Cycle, First-Strand Master Mix.

Add 4 µl of diluted T7-Oligo (dT) Primer to each sample. Incubate for 6 minutes at 70°C, and then move samples to ice and incubate for 5 minutes. Prepare sufficient Second-Cycle, Second-Strand Master Mix below, add to each sample for a total volume of 150 µl. Incubate for 2 hours at 16°C. Add 2 µl of T4 DNA Polymerase, and then incubate 10 minutes at 16°C. Then move samples to ice and incubate for 5 minutes.

Components	Volume
RNase-free Water	88 µl
5X Second Strand Reaction Mix	30 µl
10mM dNTPs	3 µl
E.coli DNA Polymerase I	4 µl
Total Volume	**125 µl**

Table 8. Preparation of Second-Cycle, Second-Strand Master Mix.

e) Cleanup of Double-Stranded cDNA

Add 600µl of cDNA Binding Buffer to the cRNA sample, mix by vortexing. Apply 500 µL of the sample to the cDNA Cleanup Spin Column sitting in a 2ml Collection Tube (supplied), and centrifuge for 1 minute at 13000 rpm. Discard flow-through. Reload the spin column with the remaining mixture and centrifuge as above. Discard flow-through and Collection Tube. Transfer spin column into a new 2ml Collection Tube. Pipet 750 µL of the cDNA Wash Buffer onto the spin column. Centrifuge for 1 minute at 13000 rpm. Discard flow-through. Open the cap of the spin column and centrifuge for 5 minutes at 13000rpm. Discard flow-through and Collection Tube. Elute the cleaned cDNA sample by transferring the RNeasy column to a new 1.5ml dark collection tube. Add 14µl cDNA Elution Buffer

directly onto the filter membrane. Wait 60 seconds at room temperature, centrifuge at 4°C for 1 minute at 13000rpm and maintain on ice.

f) Synthesis, Cleanup and Quantification of Biotin-Labeled cRNA

Transfer the needed amount of template cDNA to RNase-free microfuge tubes and add the following reaction components in the order indicated in the table below. Incubate at 37°C for 16 hours.

Components	Volume
Template cDNA	12 µl
10X IVT Labeling Buffer	4 µl
IVT Labeling NTP Mix	12 µl
IVT Labeling Enzyme Mix	4 µl
RNase-free Water	8 µl
Total Volume	**40 µl**

Table 9. Preparation of IVT Reaction Mix.

Add 60µl of RNase-free water to the IVT reaction, and then add 350µl of IVT cRNA Binding Buffer and mix well by pipetting. Add 250µl 100% ethanol and mix thoroughly by pipetting. Transfer the 700µl of the IVT cRNA Cleanup Spin Column in a 2ml collection tube. Centrifuge the sample at 4°C for 30 seconds at 13000 rpm. Transfer the RNeasy column to a new collection tube and add 500µl of IVT cRNA Wash Buffer to the column. Centrifuge the sample at 4°C for 30 seconds at 13000rpm. Discard the flow-through. Pipet 500µl 80% ethanol to the column. Centrifuge the sample at 4°C for 60 seconds at 13000rpm. Open the cap of the spin column and centrifuge for 5 minutes at 13000rpm. Discard flow-through and Collection Tube. Elute the cleaned cRNA sample by transferring the RNeasy column to a new 1.5ml dark collection tube. Add 11µl RNase-free water directly onto the spin column membrane. Centrifuge at 4°C for 1 minute at 13000rpm. Pipet 10 µl of RNase-free Water directly onto the column membrane.

Microarray Measurement tab from the NanoDrop ND-1000 Spectrophotometer was used to quantitate cRNA. Blank the instrument by pipetting 1.0µl of nuclease-free water. Take measurements of all samples and record RNA concentration values described above.

5.7.2 HYBRIDIZATION

Mix the components listed in following table for each target. Heat the hybridization cocktail to 99°C for 5 minutes; transfer it to a 45°C heat block for 5 minutes. Spin the hybridization cocktail at maximum speed in a microcentrifuge for 5 minutes to collect any insoluble material from the hybridization mixture.

Components	Volume
Fragmented and Labeled cRNA	15 µg
Control Oligonucleotide B2 (3nM)	5 µl
20X Hybridization Controls	15 µl
2X Hybridization Mix	150 µl
DMSO	30 µl
Nuclease-free water	85 µl
Total Volume	**300 µl**

Table 10. Hybridization Cocktail for Single Probe Array.

Incubate the probe array filled with Pre-Hybridization Mix at 45°C for 10 minutes with rotation. Remove the array from the hybridization oven. Vent the array with a clean pipette tip and extract the Pre-Hybridization Mix from the array with a micropipettor. Refill the array with the appropriate volume of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube. Place probe array into the hybridization oven, set to 45°C. Hybridize for 16 hours.

5.7.3 MICROARRAY WASH

Pre-warm the Gene Expression Wash Buffer 2 to 37°C over night. Take out the microarray slides, control the air bubbles. Then wash the microarray slides according to the manual under the hood.

5.7.4 SCANNING AND FEATURE EXTRACTION

Assemble the slides into an appropriate slide holder and verify scan settings for one- color scans as below. Verify the scanner status, when it's ready then start the scanning. After the scanning, generate the microarray scan images as well as QC report, and extract TIF images using Feature Extraction 9.1 software. Data analysis was performed later with Agilent GeneSpring GX10.2 software. All data were log2 transformed. A list of all the genes

included in these microarrays and the normalized data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/info/linking.html) under GEO accession number GSE26886.

5.8 IN SITU HYBRIDIZATION

A 148 bp fragment located at the 3 terminal end of human WDR66 gene (NM144668) was subcloned into the pBluescript II vector pBS-27.16 using primer pair forward: 5'-CAACCTgCTCCgTCAAA-3' and reverse: 5'-TAAACATTCCTggTAACTTCAC-3'. The linearized plasmid was used as a template for the synthesis of antisense probes. The probe was labeled by digoxigenin / dUTP with a DIG/RNA labelling kit. The quality and quantity of the probe were confirmed by gel electrophoresis before used for ISH. Digoxigenin-labeled probe was applied to 5um dewaxed FFPE sections, and hybridized at 65°C overnight in a humid chamber. After 3 washes to remove the nonspecific binding or unbound probes, digoxigenin-labeled probe was detected using the alkaline phosphatase method.

5.9 WESTERN BLOTTING ANALYSIS

Total cell extracts were obtained and cell lysate containing 50μg of protein was separated on 10% SDS-polyacrylamide gel and then blotted onto polyvinylidene difluoride (PVDF) membranes. Primary antibody for vimentin detection was mouse monoclonal anti-human vimentin antibody. Primary antibody for occludin detection was rabbit polyclonal anti-human occludin antibody. ß-actin was used as loading control. Signals were detected using ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Images were scanned by FujiFilm LAS-1000 (FujiFilm, Düsseldorf, Germany).

5.10 CELL MOTILITY ASSAY

Transwell chambers were used in the cell mobility experiments. Cells were inoculated into the upper compartment of the Transwell chambers at a concentration of 1×10^5 cells/mL and 100 µl/well. The medium for the experimental and control groups was added into the lower compartment of the Transwell chambers (500 µL/well). The cells were cultured at 37°C for 10 hours. Cells that did not penetrate the polycarbonate membrane at the bottom of the chamber were wiped off with cotton stickers. Nonmigrated cells were removed, and the migrated cells were detected by qRT-PCR.

5.11 WOUND HEALING ASSAY

Wound healing assays were performed in triplicates using cytoselect 24-well wound healing assay (Cell Biolabs, Inc.) according to the manufacturer's instructions. Briefly, cells were cultured in 24-well plate until they form a monolayer around the insert. The insert is removed, leaving a precise 0.9 mm open "wound field" between the cells. Cells were monitored at this point for migration and proliferation into the wound field. Wound healing was visualized by comparing photographs taken at the time points of t0 (insert removed) and t8 (8 hours after).

5.12 APOPTOSIS ANALYSIS

Staurosporine was dissolved in RPMI 1640 to a final concentration of 10 nM, 50 nM, 100nM and 500 nM. 5-Fluorouracil was dissolved in RPMI 1640 to a final concentration of 0.1 mg/ml, 1mg/ml and 10mg/ml. For the control experiments, cells were incubated in RPMI 1640 medium containing 10% FCS (without staurosporine and 5-Fluorouracil).

Cells were plated in black 96-well Falcone cell-culture plates with optical bottoms, as described above, and incubated either with staurosporine or with 5-Fluorouracil. The calcein was added to each sample well before the fluorescence measurement was carried

out using a POLARstar OMEGA reader (BMC Labtech). Emission and excitation filters were 485 and 520 nm. The results were analyzed by MARS data analysis software.

5.13 STATISTICAL ANALYSIS

Statistical analysis was done using GraphPad Prism version 5 for Windows (GraphPad Software) and SPSS version 13 for Windows (SPSS, Chicago, IL, USA) as follows: GraphPad Prism, Unpaired t test with Welch's correction of quantitative real-time RT-PCR measurements of WDR66 in patient samples and of gene expression measurements in the validation cohort; nonparametric Mann-Whitney U test of cell numbers, motility assay, and cell wound assay after knockdown of WDR66; SPSS, Kaplan-Meier survival analysis and log-rank statistics, cut-point analysis of quantitative real-time RT-PCR measurements of WDR66 in patient samples using maximally selected rank statistics to determine the value separating a group into two groups with the most significant difference when used as a cut-point; grouping of patients according to median of quantitative real-time RT-PCR measurements was done as follows: WDR66 $\leqslant$ 125, WDR66 low; WDR66 > 125, WDR66 high; the stratified Cox-regression model was used to determine prognostic factors in a multivariate analysis with WDR66 dichotomized at the previously determined cut-points.

6. RESULTS

6.1 WDR66 IS SPECIFICALLY AND HIGHLY EXPRESSED IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA.

One aim of this project was the WDR66 gene expression analysis in esophageal squamous cell carcinoma patient cohort. For this purpose, 10 cancer tissue samples and 18 normal esophageal squamous epithelium samples were selected from the tumor bank of the Charité Comprehensive Cancer Center. As mentioned before, we applied the laser capture microdissection (LMD) technique to the specimens to make sure that only epithelial cells were studied. A number of genes differentially expressed between esophageal squamous cell carcinoma samples and normal esophageal squamous epithelium samples were identified. The probe set with the highest fold change and lowest p-value represented the WDR66 transcript ($P < 0.0001$) (Figure 13).

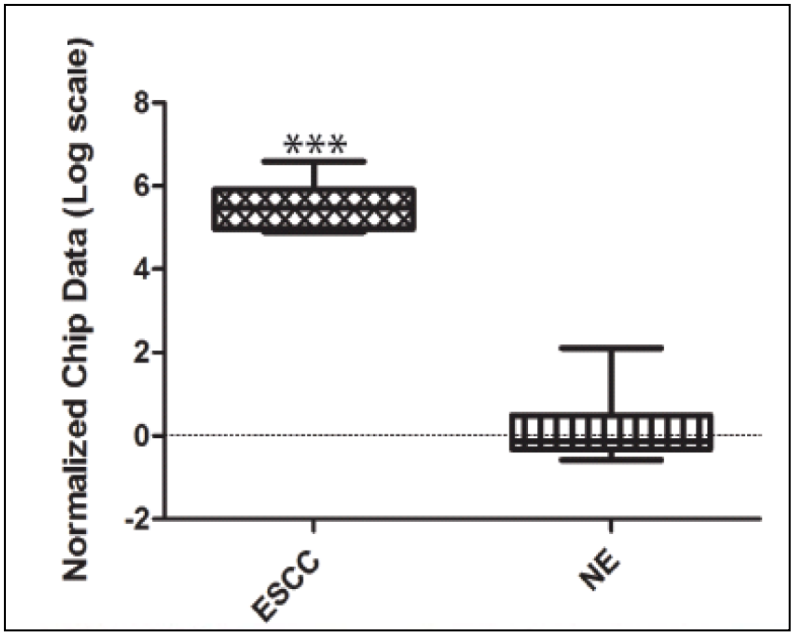

Figure 13. mRNA expression of the WDR66 gene was determined by microarray analysis. Microarray analysis was performed on 18 healthy normal esophageal epithelium (NE) and 10 esophageal squamous cell carcinoma (ESCC) samples. Gene expression is presented as normalized (log2 scale) signal intensity of the WDR66 gene. The WDR66 gene is significantly differentially expressed in ESCC (corrected p-value < 0.0001). Expression level of WDR66 gene is low in NE but high in ESCC cases. The horizontal axis depicts the patient groups ESCC and NE. (* P < 0.001)**

As a validation study, WDR66 expression was examined by quantitative real-time polymerase chain reaction (qPCR) in an independent cohort consisting of 71 specimens including esophageal squamous cell carcinoma (ESCC) (n=25), normal esophageal squamous epithelium (NE) (n=11), esophageal adenocarcinoma (EAC) (n=13), gastric adenocarcinoma (GAC) (n=15) and colorectal cancers (CRC) (n=7). We found that WDR66 was highly expressed in 96% of ESCC patients (Figure 14). Confirming our previous results from the microarray study, WDR66 expression was found to be significantly higher in ESCC compared to NE as well as the other three cancer types checked in this cohort ($P < 0.0001$).

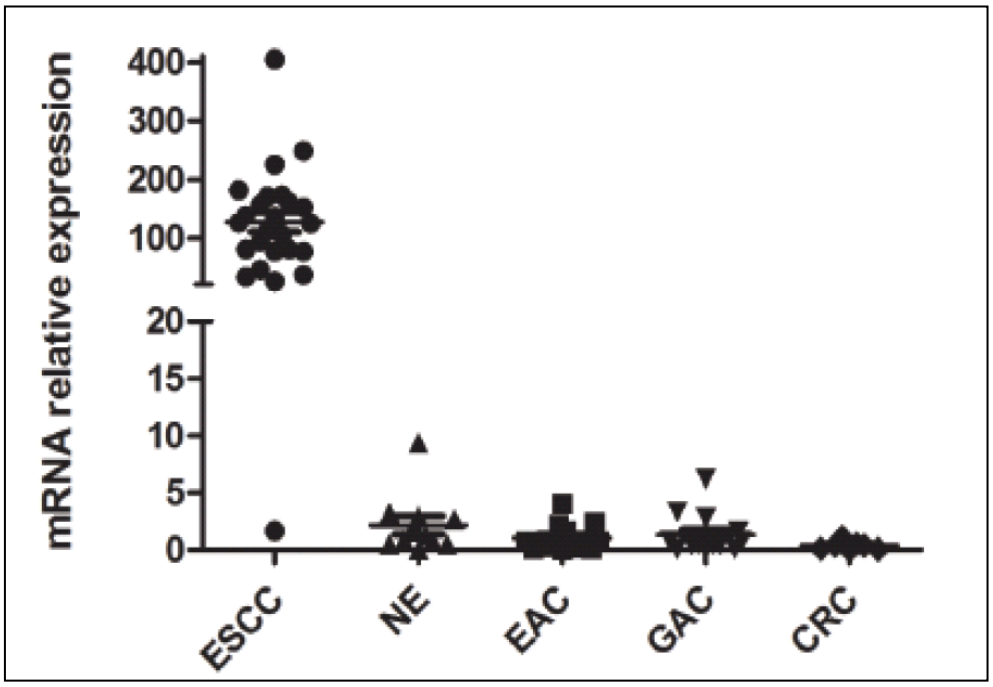

Figure 14. Relative mRNA expression of the WDR66 gene in an independent validation cohort determined by qPCR. The mRNA abundance analysis was performed on 71 specimens including ESCC (n=25), NE (n=11), EAC (n=13), GAC (n=15) and CRC (n=7) samples by means of quantitative real-time PCR. Quantitation was done relative to the transcript of GAPDH. Significance in differential expression of individual gene between groups was calculated (p-value < 0.001). Results showed that WDR66 gene expression level is highest in ESCC and low to absent in NE or other carcinomas. On the horizontal axis patient groups ESCC, NE, EAC, GAC and CRC are depicted.

Immunohistochemical localization of WDR66 was not carried out because none of the WDR66 antibodies available allowed detecting a specific protein band on western blots.

Therefore, the presence of WDR66-specific mRNA was probed by in-situ hybridization using single-stranded RNA probes of the WDR66 gene in 4% PFA-fixed paraffin-embedded esophageal tissues. WDR66 transcription (positive staining) was specifically detected in the esophageal squamous carcinoma cells but not in normal squamous epithelia (Figure 15).

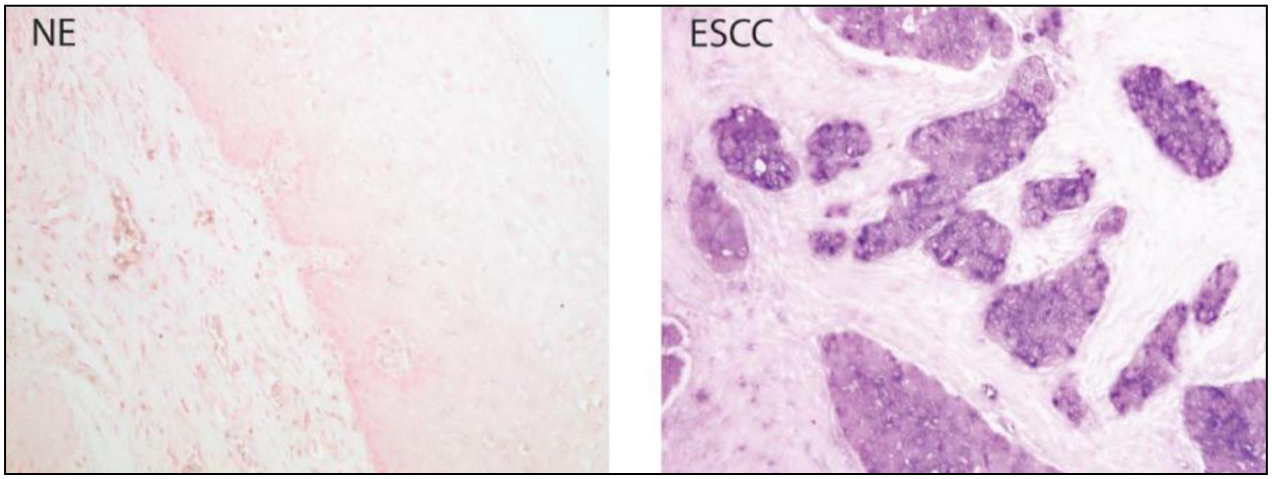

Figure 15. WDR66 gene is highly expressed in ESCC epithelium according to in situ hybridization. In situ hybridization was done using anti-sense probes of human WDR66 gene in FFPE sections of esophageal specimens. Signals for WDR66 transcripts were observed specifically in esophageal squamous cell carcinoma (ESCC, right), but not in normal squamous epithelium (NE, left).

Furthermore, WDR66 expression was examined in 14 human cell-lines and 20 normal human tissues by qPCR. Expression of WDR66 gene was abundantly expressed only in the human esophageal squamous cell line KYSE520, but not expressed in any other human cell line, such as OE33, SW480, HT29, HCT116, LS174T, Caco2, HL60, HEK293, Daudi, Capan1, MCF7, MDA-MB231 or MDA-MB435 (Figure 16). Among 20 normal human tissues examined by qPCR, WDR66 was most abundantly expressed in the testis (Figure 17). Thus, our data suggest that WDR66 might be a cancer / testis antigen.

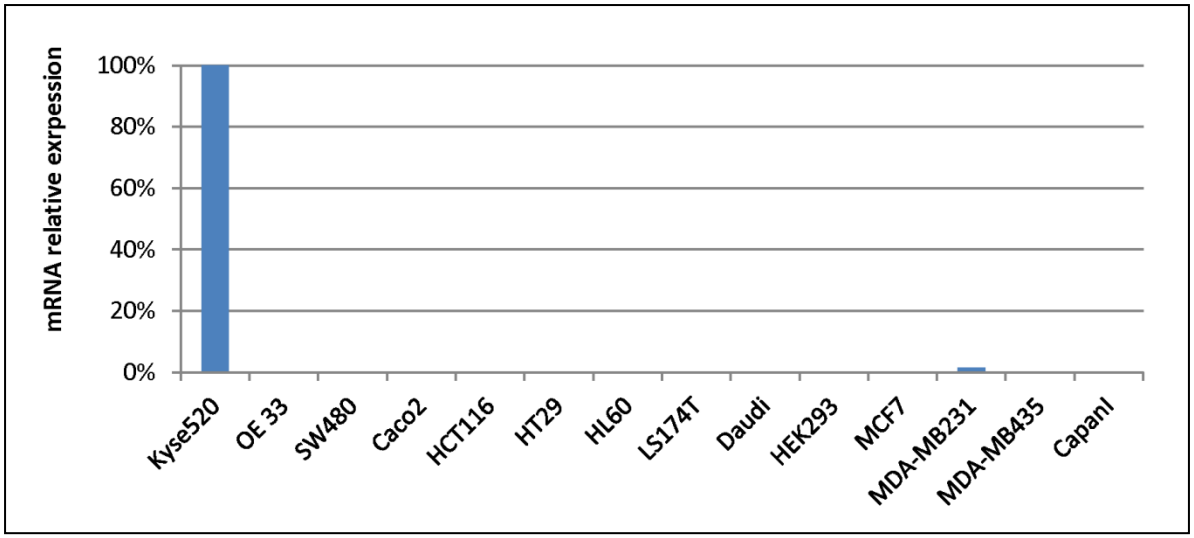

Figure 16. WDR66 expression level in various human cell lines. WDR66 expression was examined by quantitative real-time PCR in 14 cell lines cultivated from different human carcinomas. The expression was quantified relative to human esophageal squamous carcinoma cell line KYSE520.

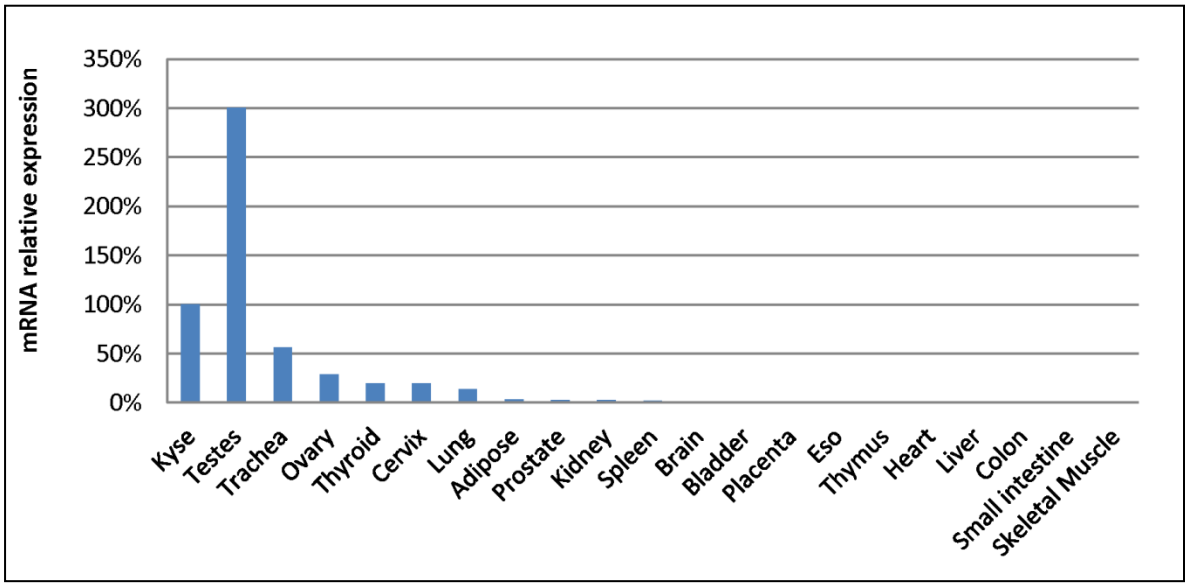

Figure 17. Tissue-specific expression of WDR66 gene in various human normal tissues. Quantitative real-time PCR analysis of WDR66 expression levels in 20 human normal tissues (FirstChoice ® Human Total RNA Survey Panel). WDR66 gene is preferentially expressed in testis. Gene level was quantified relative to the expression in ESCC cell line KYSE520.

6.2 High expression of WDR66 correlates with poor survival outcome in ESCC

In order to test if WDR66 expression correlates with prognostic markers in a separate validation set of ESCC examples, we determined WDR66 expression in an independent set of n=25 ESCC examples using qRT-PCR. Using WDR66 expression value as measured by quantitative real-time PCR we set up two groups: WDR66 low group (WDR66≤125) and WDR66 high group (WDR66>125). High expression of WDR66 RNA was found to be a significant prognostic factor with regard to cancer-related survival (P = 0.031; Figure 18). When analyzed with regard to various clinicopathological parameters, such as gender (P = 0.804), age (P = 0.432), tumor differentiation (P =0.032), pT factor (P = 0.234), lymph node metastasis (P = 0.545), distant metastasis (P = 0.543) and TNM stage (P = 0.002), multivariate Cox regression analysis revealed that WDR66 expression remained an independent prognostic factor (P = 0.042; Table 11)

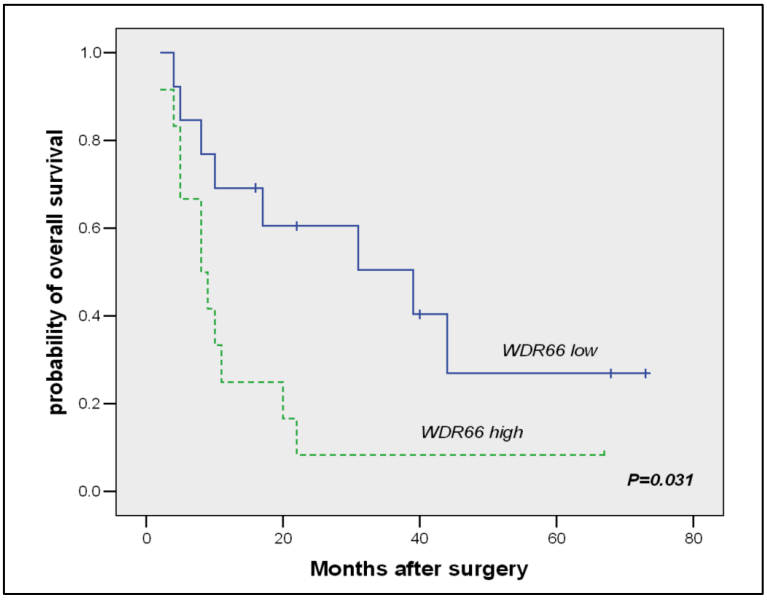

Figure 18. High WDR66 mRNA expression is associated with poor survival in ESCC patients. Kaplan-Meier analysis of survival of grouped according to WDR66 expression as measured by quantitative real-time RT-PCR. Grouping of patients according to median value of WDR66 mRNA expression by qRT-PCR measurements was done as follows: WDR66 ⩽ 125, WDR66 low group (n=12); WDR66 > 125, WDR66 high group (n=13). After choosing an optimal cut-point, analysis for WDR66 was done using maximally selected rank statistics. The group with patients expressing WDR66 at low levels showed a significantly better overall survival compared with the group with high levels of WDR66 expression (P = 0.031; log rank).

Variables	Hazard ratio	95% CI	*P*
WDR66 (high vs low)	**2.644**	**1.034-6.758**	**0.042**
Gender (male vs female)	1.15	0.380-3.48	0.804
Age, y (<60 vs ≥60)	1.46	0.569-3.748	0.432
Tumor differentiation (high grade vs low or Intermediate grade)	**2.763**	**1.093-6.983**	**0.032**
pT factor (pT3 or pT4 vs pT1 or pT2)	2.133	0.613-7.420	0.234
Lymph node metastasis (yes vs no)	1.575	0.362-6.852	0.545
Distant metastasis (yes vs no)	1.376	0.492-3.846	0.543
TNM stage (I or II vs III or IV)	**7.711**	**2.181-27.259**	**0.002**

Table 11. Cox regression analysis for factors possibly influencing disease-specific survival in patients with ESCC in our cohort.

6.3 KNOCKDOWN OF WDR66 IN KYSE520 EFFECTED VIM AND OCLN EXPRESSION IN VITRO

In order to learn more about the function of WDR66, RNA interference was used to silence its expression in KYSE520 cells, a human esophageal squamous cell carcinoma cell line which highly expressed WDR66. Subsequently, a microarray expression analysis was performed in order to identify the genes affected by WDR66 knockdown. A total of 699 genes were identified based on a two-fold change expression difference with p-value of $P < 0.001$. In an approach to link the observed gene expression profile to gene function, these 699 differentially expressed genes were subjected to gene ontology (GO) analysis. Functional enrichment analysis identified 10 GO terms to be significantly associated with the WDR66 knockdown. All these 10 GO terms are membrane related (Table 12).

GO ID	GO ACCESSION	GO Term	p-value	corrected p-value
3634	GO:0005886	plasma membrane	2,78E-11	6,29E-07
8544	GO:0016020	membrane	3,90E-09	4,42E-05
18816	GO:0044425	membrane part	6,93E-08	5,24E-04
4699	GO:0007155	cell adhesion	1,55E-07	7,29E-04
11867	GO:0022610	biological adhesion	1,61E-07	7,29E-04
13052	GO:0031224	intrinsic to membrane	4,18E-07	1,58E-03
8545	GO:0016021	integral to membrane	7,65E-07	2,48E-03
6433	GO:0009615	response to virus	9,31E-07	2,64E-03
18849	GO:0044459	plasma membrane part	1,39E-06	3,50E-03
3653	GO:0005911	intercellular junction	1,30E-05	2,27E-02

Table 12. Significantly enriched Gene Ontology (GO) terms identified for genes differentially expressed in siWDR66 KYSE520 cells. GO analysis was performed using GeneSpring. The 10 GO terms with the significant corrected P-value (FDR false discovery rate corrected for multiple testing) are depicted sorted by p-Value (non-corrected)

This microarray data revealed an association of WDR66 with membrane function. We sought to determine if knockdown of WDR66 would alter the epithelial-to-mesenchymal transition (EMT) in KYSE520. We chose vimentin (VIM), which is used as a marker of cells undergoing EMT, and occludin (OCLN), an important tight junction protein as targets.

Gene expression level of VIM was significantly down-regulated whereas OCLN expression was significantly higher in cells treated with WDR66 siRNA in comparison to NTC (KYSE520) and Allstar (negative control siRNA) (corrected p-value: VIM, P = 0.0286; OCLN, P =0.0186) (Figure 19). Detection of VIM and OCLN protein by immunoblotting of KYSE520 cells treated with WDR66 siRNA in comparison to NTC (KYSE520) and Allstar (negative control siRNA). VIM expression was significantly down-regulated while OCLN was significantly higher expressed in cells treated with WDR66 siRNA (Figure 20).

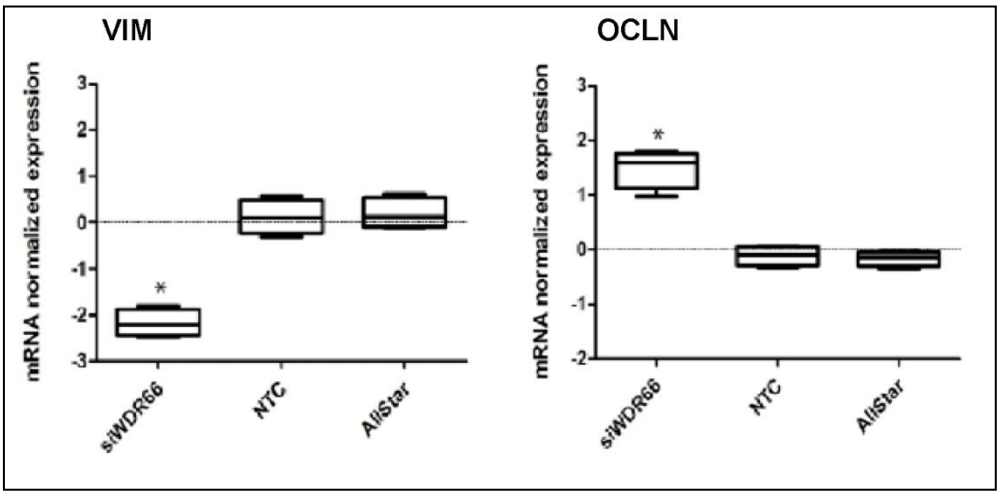

Figure 19. Knockdown of WDR66 affects mRNA expression of VIM and OCLN. Gene expression is presented as normalized (log scale) signal intensity for mRNA expression levels of VIM and OCLN gene. Gene expression level of VIM was significantly down regulated whereas O CLN expression was significantly higher in cells treated with WDR66 siRNA in comparison to NTC (KYSE520) and Allstar (negative control siRNA) (corrected p-value: VIM P = 0.0286; OCLN P = 0.0186). Data are representative of four independent experiments. (∗P < 0.05).

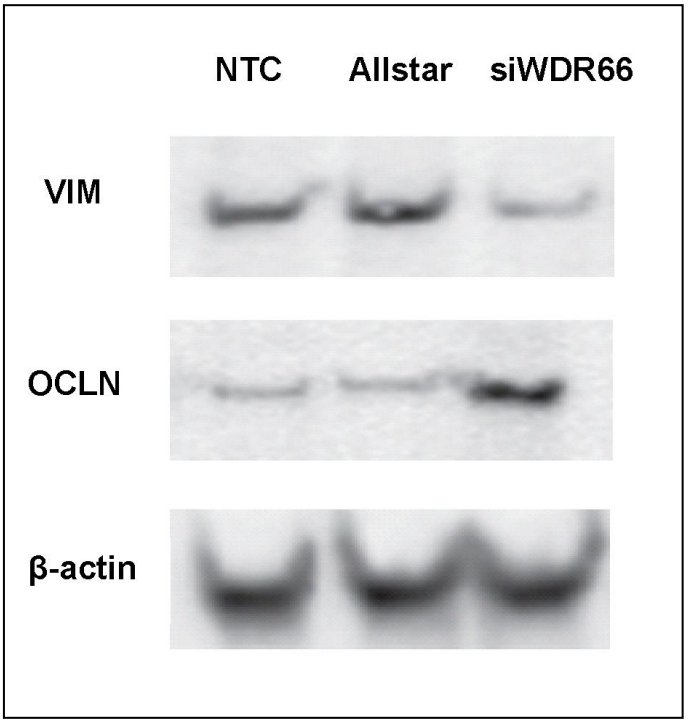

Figure 20. Detection of vimentin and occludin protein by immunoblotting of KYSE520 cells treated with WDR66 siRNA in comparison to NTC (KYSE520) and Allstar (negative control siRNA). β-actin was used as loading control. Vimentin expression was significantly down-regulated while occludin was significantly higher expressed in cells treated with WDR66 siRNA.

Meanwhile, we also checked the expression of VIM and OCLN in ESCC patients of our cohort and found that VIM was highly expressed (P = 0.0008) while OCLN was less expressed (P < 0.0001) in ESCC specimens in comparison to NE (Figure 21).

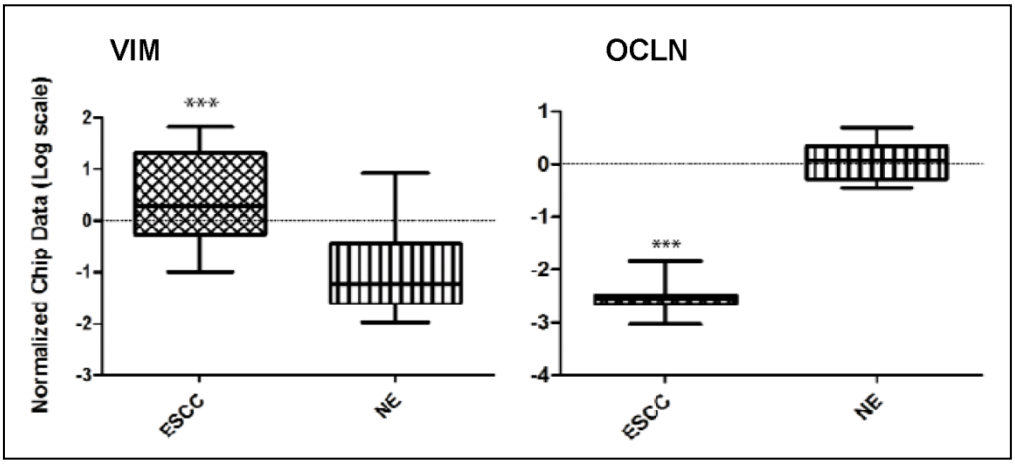

Figure 21. mRNA expression of the VIM and OCLN gene in the original training cohort determined by microarray analysis. Microarray data analysis was performed on 18 healthy normal esophageal epithelium (NE) and 10 esophageal squamous cell carcinoma (ESCC) samples. Gene expression is presented as normalized (log2 scale) signal intensity of the genes of interest. VIM and OCLN are significantly differentially expressed in ESCC compared with NE (corrected p-value: VIM, P = 0.0008; OCLN, P < 0.0001). VIM expression level is low in NE but high in ESCC cases, whereas OCLN expression is high in NE but low in ESCC cases. The horizontal axis depicts the patient groups ESCC and NE. (* P < 0.001)**

6.4 KNOCKDOWN OF WDR66 IN KYSE520 CELLS AFFECTS CELL MOTILITY AND RESULTS IN GROWTH SUPPRESSION.

Due to the effect of WDR66 on the expression of VIM, an important EMT marker which plays a central role in the reversible trans-differentiation and OCLN, an adhesion molecules that is a constituent of tight junctions, we hypothesized that WDR66 may regulate cell motility of esophageal squamous cancer cells.

WDR66 was knockdown in the human squamous cell carcinoma cell line KYSE520 by RNA interference. Transfection efficiency was evaluated by qPCR. Cell migration assays showed that KYSE520 cells, which had been transfected with WDR66 siRNA, displayed a

motility capacity of only 35% compared to the cells transfected with control siRNA (AllStar) after 16 hours (Figure 22).

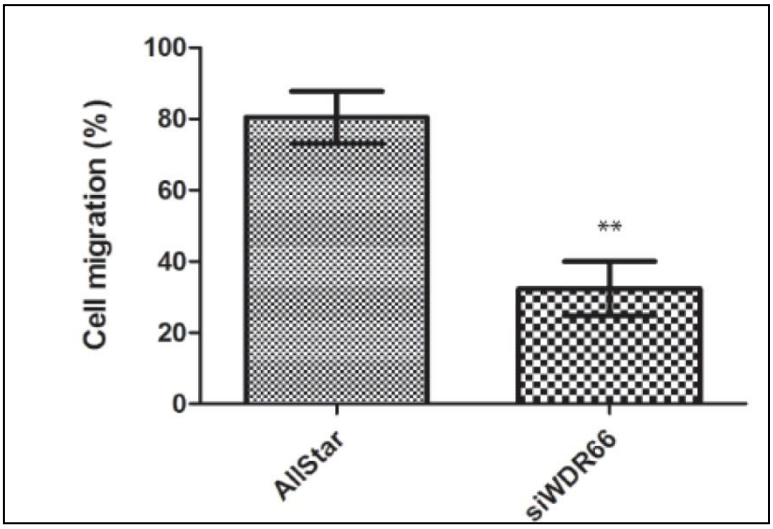

Figure 22. Cell motility assays showed that knockdown of WDR66 reduced cell migration after 16 hours. About 35% of the siWDR66 cells were migrated in comparison to the mock control cells. The differences between siWDR66 and Allstar (negative control siRNA) cells were significant (P = 0.0032) using the paired t test. The data shown are representative of three independent experiments each done in quadruplicate.

Moreover, we found that introduction of siWDR66 markedly suppressed growth of KYSE520 in comparison to control cells (Figure 23).

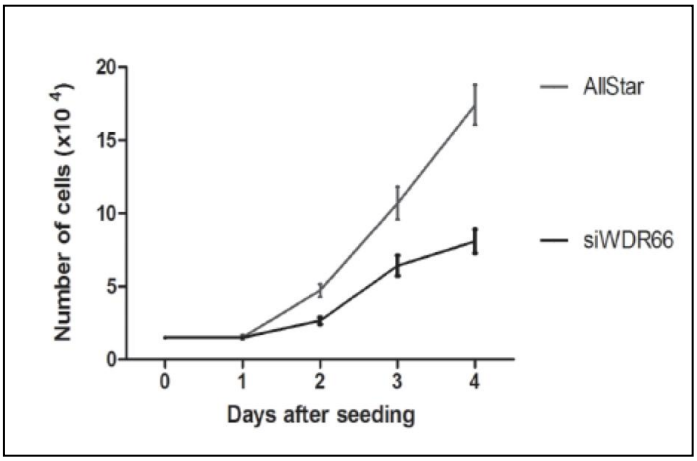

Figure 23. Knockdown of WDR66 leads to suppression of cell growth. A total of 1.5x104 cells were seeded at day 0. WDR66 knockdown cells grew slower than cells treated with Allstar (negative control siRNA). The differences between siWDR66 and Allstar (negative control siRNA) treated cells were highly significant (P = 0. 0098).

In order to visualize the involvement of WDR66 in cell migration and proliferation, a wound-healing assay was carried out. The insert was removed at defined time points of scratching and the results were recorded by taking pictures (Figure 24). Thus, our data suggest that WDR66 promotes cell proliferation and affects cell motility.

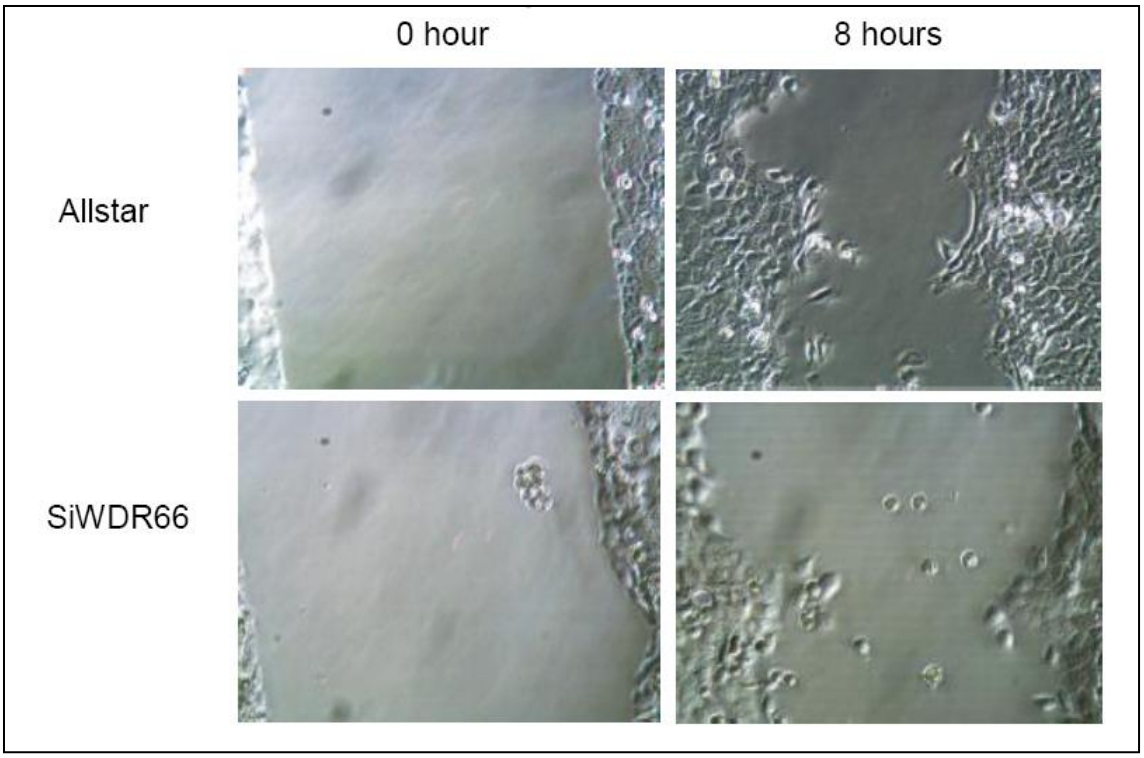

Figure 24. Wound-healing assays show that knockdown of WDR66 reduces cell motility. Representative images are shown. Images are taken immediately after insert was removed and 8 hours later. Original magnification is x400.

6.5 WDR66 AND CELL APOPTOSIS ANALYSIS

6.5.1 STAUROSPORINE INDUCED THE APOPTOTIC DEATH OF KYSE520

Staurosporine is a prototypical ATP-competitive kinase inhibitor in that it binds to many kinases with high affinity, though with little selectivity. This lack of specificity has precluded its clinical use, but has made it a valuable research tool. In research, staurosporine is used to induce apoptosis.

In order to address the ability of staurosporine to induce cell death, we first investigated the effect of staurosporine on cell apoptosis. After 8 hours incubation, loss of viability occurred following staurosporine exposure in a time-dependent manner (Figure 25A). The incubation with staurosporine (0–500 nM) caused a marked decrease in cell viability in a dose-dependent manner (Figure 25B).

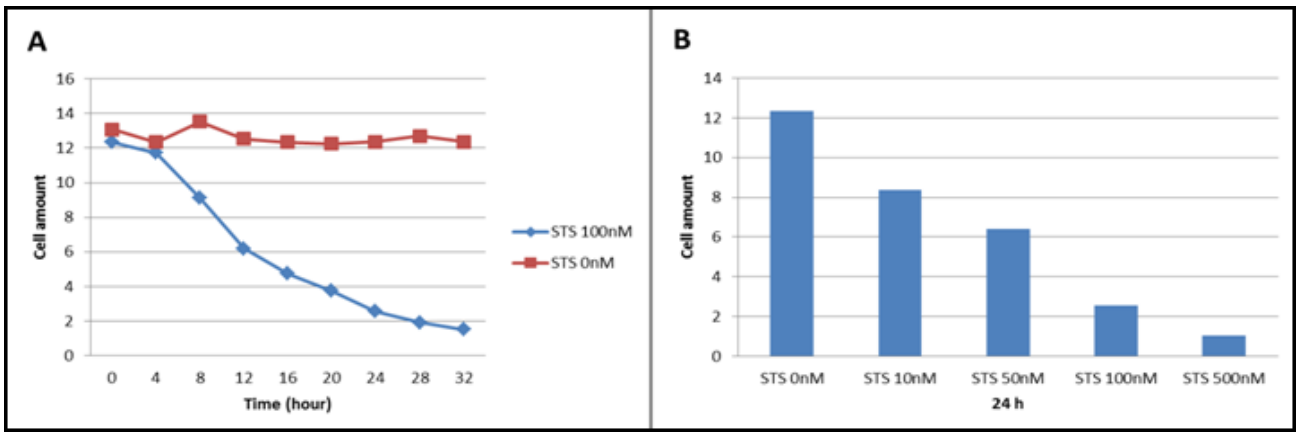

Figure 25. Staurosporine(STS) induces cell apoptosis in KYSE520 cell line. Cell apoptosis was assessed by fluorescence measurement using POLARstar Omega.

A: KYSE520 cells were subjected to 100nM staurosporine for 0, 4, 8, 12, 16, 20, 24, 28 or 32 h.

B: Cells were incubated with 0, 10, 50, 100 or 500 nM staurosporine for 24 h

6.5.2 The effect of WDR66 on the apoptosis induced by Staurosporine

We determined whether WDR66 knockdown might induce cell apoptosis by the addition of staurosporine (100 nM). WDR66 knockdown induced KYSE520 apoptosis by the incubation with staurosporine at 8 hours, reached a maximum at 12 hours, and continued the effect until 32 hours (Figure 26A). At 12 hours, WDR66 knockdown induced KYSE520 cell apoptosis by the incubation with staurosporine (0, 10, 50 and 100 nM) (Figure 26B).

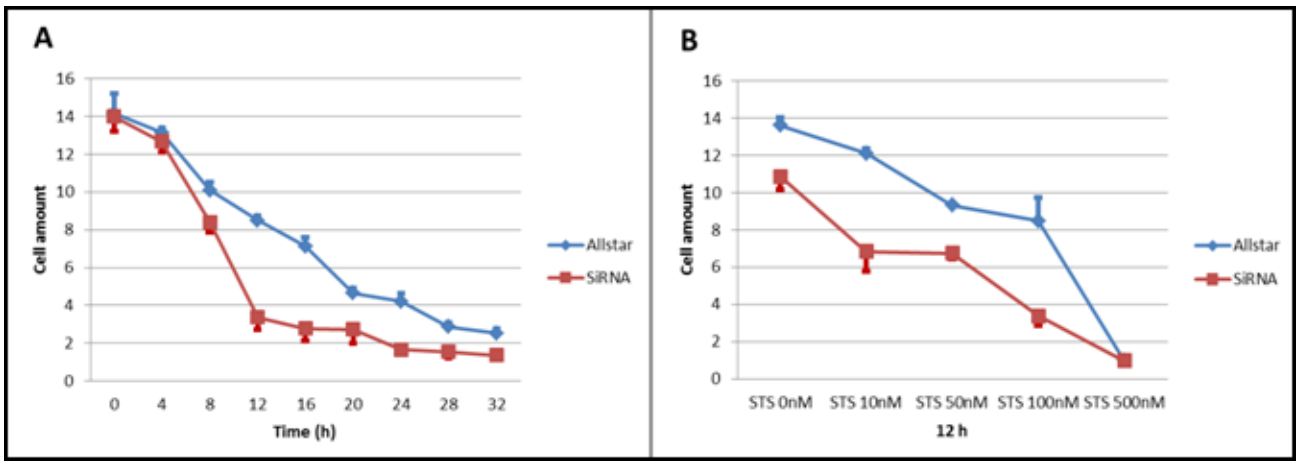

Figure 26. WDR66 knockdown induced KYSE520 cell apoptosis by the incubation of staurosporine. Cell apoptosis was assessed by fluorescence measurement using POLARstar Omega.

A: Knockdown WDR66 induced KYSE520 cell apoptosis by the incubation with staurosporine (100 nM) at 8h, reached a maximum at 12h, and continued the effect until 32h. The differences between siWDR66 and control siRNA (AllStar) cells were significant at 8h (P = 0.015), at 12h (P = 0.001), at 16h (P = 0.001), at 20h (P = 0.012), at 24h (P = 0.007), at 28h (P = 0.019) and at 32h (P = 0.03) using the paired t test. The data shown are representative of three independent experiments each done in quadruplicate.

B: At 12h, WDR66 knockdown induced KYSE520 cell apoptosis by the incubation with staurosporine 0nM (P = 0.005), staurosporine 10nM (P = 0.001), staurosporine 50nM (P = 0.003), staurosporine 100nM (P = 0.001). The data shown are representative of three independent experiments each done in quadruplicate.

6.5.3 5-FLUOROURACIL INDUCED THE APOPTOTIC DEATH OF KYSE520

The chemotherapy agent 5-Fluorouracil (5-FU), which has been used against cancer for about 40 years, acts in several ways, but principally as a thymidylate synthase inhibitor. We first investigated the effect of 5-FU on KYSE520 cell apoptosis. Some of its principal uses are in esophageal cancer, colorectal cancer, and pancreatic cancer, in which it has been the established form of chemotherapy for decades (platinum-containing drugs approved for human use in the US since 1978 are also very well established).

48 hours later, loss of viability occurred following 5-FU exposure in a time-dependent manner (Figure 27A). The incubation with 5-FU (0–10 mg/ml) caused a marked decrease in cell viability in a dose-dependent manner (Figure 27B).

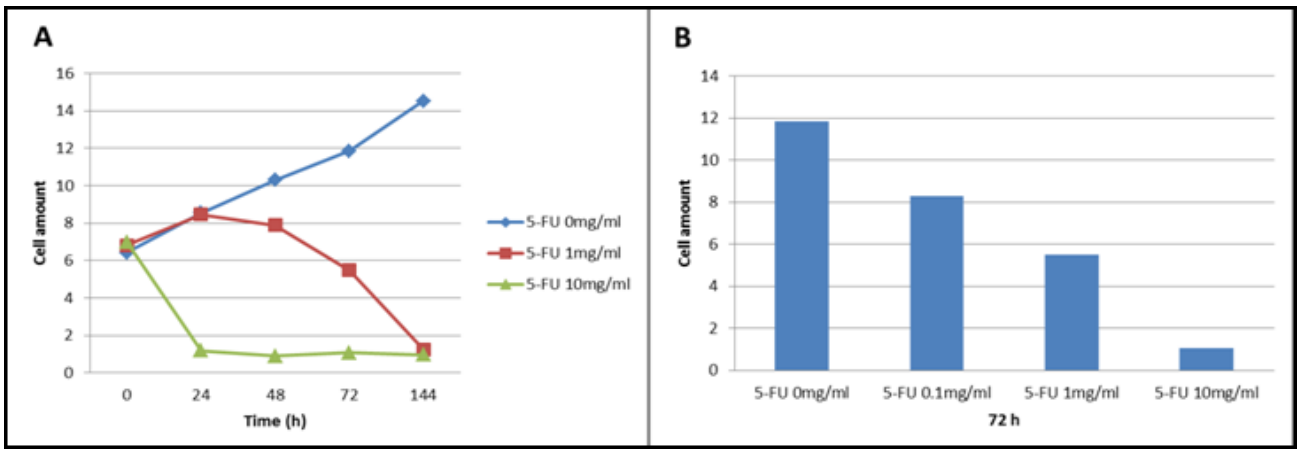

Figure 27. 5-Fluorouracil (5-FU) induces cell apoptosis in KYSE520 cell line. Cell apoptosis was assessed by fluorescence measurement using POLARstar Omega.

A: KYSE520 cells were subjected to 1mg/ml 5-FU and 10mg/ml 5-FU for 0, 24, 48, 72 or 144 h.

B: Cells were incubated with 0, 0.1, 1 or 10 mg/ml 5-FU for 72 h.

6.5.4 THE EFFECT OF WDR66 ON THE APOPTOSIS INDUCED BY 5-FU

We determined whether WDR66 knockdown might induce cell apoptosis by the addition of 5-FU (1 mg/ml). WDR66 knockdown induced KYSE520 cell apoptosis by the incubation with 5-FU at 24 hours, 48 hours and 72 hours (Figure 28A). At 48 hours, WDR66 knockdown induced KYSE520 cell apoptosis by the incubation with 5-FU (0, 0.1 and 1 mg/ml) (Figure 28B).

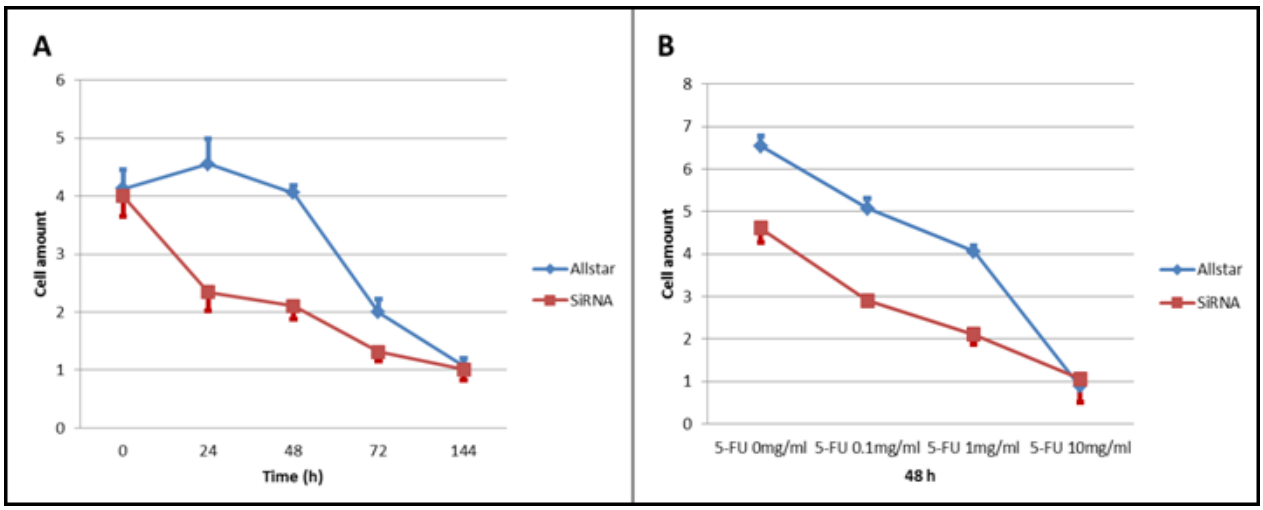

Figure 28. WDR66 knockdown induced KYSE520 cell apoptosis by the incubation with 5-Fluorouracil. Cell apoptosis was assessed by fluorescence measurement using POLARstar Omega.

A: Knockdown WDR66 induced KYSE520 cell apoptosis by the incubation 5-FU(1mg/ml) at 24 hours, 48 hours and 72 hours. The differences between siWDR66 and control siRNA (AllStar) cells were significant at 24h (P = 0.001), at 48h (P = 0.001) and at 72h (P = 0.008) using the paired t test. The data shown are representative of three independent experiments and each done in quadruplicate.

B: At 48 hours, WDR66 knockdown induced KYSE520 apoptosis by the incubation with 5-FU 0mg/ml (P = 0.001), 5-FU 0.1mg/ml (P = 0.001) and 5-FU 1mg/ml (P = 0.001). The data shown are representative of three independent experiments and each done in quadruplicate.

7. DISCUSSION

7.1 WDR GENE FAMILY AND CANCER

WD-repeat proteins are made up of highly conserved repeating units usually ending with Trp-Asp. They regulate cellular functions, such as cell division, cell-fate determination, gene transcription and transmembrane signaling [11].

Among the WD-repeat proteins, endonuclein containing five WD-repeat domains was shown to be up-regulated in pancreatic cancer [19]. The expression of human beta-transducing repeat-containing protein (BTRC), which contains one F-box and seven WD-repeats, targeted at epithelial cells under tissue specific promoter in BTRC deficient (-/-) female mice, promoted the development of mammary tumors [20]. WD repeat-containing protein 16 (WDRPUH) encoding a protein containing 11 highly conserved WD-repeat domains was also shown to be up-regulated in human hepatocellular carcinomas and involved in the promotion of cell proliferation [21]. The WDR3 gene, maps in the 1p12 region, was revealed that can increase the risk of thyroid cancer in a case–control study. Both messenger RNA transcription and protein expression of WDR3 were then modified in human thyroid cancer cells [22]. Sato et al. screened for genes that were overexpressed in a large proportion of lung and esophageal carcinomas using a cDNA microarray. A gene encoding WDHD1, a WD repeat and high-mobility group box DNA binding protein 1, was selected as a novel biomarker and therapeutic target for lung and esophageal cancers which is likely to play a role as a cell cycle regulator and a downstream molecule in the phosphoinositide 3-kinase/AKT pathway [23]. F-box and WD repeat domain-containing 7 (FBW7) has been characterized as an onco-suppressor protein in human cancers by promoting the degradation of various oncoproteins, through which FBW7 regulates cellular proliferation, differentiation and causes genetic instability [24]. Tu et al. determined that the mRNA and protein expression of Fbxw7 was significantly down-regulated in hepatocellular carcinoma tumor tissues compared to normal tumor-adjacent tissues. Fbxw7 protein was expressed at significantly lower levels in patients with high histological grade and advanced tumor-node-metastasis stage. Fbxw7 protein expression was negatively correlated with c-Myc, cyclin E and p53 and may have prognostic potential through the enhanced function of

cell cycle regulatory proteins [25]. WD repeat domain 1 was identified and verified to be significantly up-regulated in the highly metastatic gallbladder carcinoma cell line compared to the poorly metastatic cell line by one-dimensional western blotting and semiquantitative reverse transcriptase polymerase chain reaction analysis [26]. WDR11, which is frequently deleted in gliomas and tumors of other tissues, strongly suggests that it is a candidate gene for the tumor suppressor locus in this region [27].

7.2 WDR66, A CANCER / TESTIS ANTIGEN

Among 20 normal human tissues examined by qRT-PCR, WDR66 expression was found more abundantly in the testis than ESCC. Thus, our data suggest that WDR66 might be a cancer / testis antigen.

Cancer-testis (CT) genes, normally expressed in germ line cells but also activated in a wide range of cancer types, often encode antigens in cancer patients [28]. Testis is an immune-privileged site as a result of a blood barrier and lack of HLA class I expression on the surface of germ cells [29]. Hence, if testis-specific genes are expressed in other tissues, they can be immunogenic. In recent years, studies on advanced tumors have revealed an immunoediting of tumor cells by altering their phenotype to escape immunoresponse [30]. Thus, expression of some CT genes in a high percentage of esophageal tumors makes them potential targets for immunotherapy, like LAGE1, MAGE-A4 and NY-ESO-1 [31].

NY-ESO-1, a highly immunogenic, prototypical protein marker, is limited in expression to a wide variety of cancer types but not in normal tissue, with the exception of the immune-privileged testes, and has been thoroughly investigated as a target for immunotherapy in cancer patients [32-35]. Immunotherapy of various cancers against NY-ESO-1 showed promising clinical results. Hunder et al. developed an in vitro method for isolating and expanding autologous CD4+ T-cell clones with specificity for NY-ESO-1. Then these cells were infused into a patient with refractory metastatic melanoma and showed that the transferred CD4+ T cells mediated a sustained clinical remission and led to endogenous responses against melanoma antigens [32]. Although this type of approach will not always work due to the variety of immunosuppressive mechanisms of cancer to defeat potentially

effective immune responses, this case report underscored the remarkable potential of the immune system to eradicate cancer [36]. Junqueira et al. demonstrated that vaccine formulations containing NY-ESO-1 are efficient immunological adjuvants which resulted in a significant delay in the growth of the B16F10 melanoma cell line [33]. Weide et al. analysed the prognostic relevance of circulating T cells responding to NY-ESO-1 in patients with melanoma with distant metastasis and found that NY-ESO-1-responsive T cells were detected in 70% of patients surviving longer than 18 months and in 50% of long-term survivors. In contrast, the proportion was only 22% for NY-ESO-1 in those who died within 6 months. These findings revealed that the presence of circulating T cells responding to NY-ESO-1 had strong independent prognostic impact on survival in advanced melanoma and supported the therapeutic relevance of NY-ESO-1 as targets for immunotherapy [34].

ECT2 (epithelial cell transforming sequence-2) is another type of cancer / testis antigen identified in lung and esophageal cancer progression, which is likely to be a prognostic biomarker in the clinic, and the expression of ECT2 promoted cellular invasive activity [37]. Another WD-repeat protein, WDRPUH, also showed cancer / testis specific expression [21].

In contrast to most cancer / testis antigens, which are expressed only in a small group of tumor patients (among 10-40% [38]), we found that WDR66 is specifically and highly expressed in 96% of ESCC patients. Furthermore, expression of WDR66 gene was abundantly expressed only in the human esophageal squamous cell carcinoma cell line KYSE520, but not in any other human cell line.

Taken together, as a candidate CT gene, WDR66 may play a role in future therapeutic approach of ESCC. The low efficiency of ESCC detection in early stages before the invasion to surrounding organs known as an advanced stage remains the main reason for mortality of ESCC. It is therefore important to gain a better understanding of the underlying molecular pathways of these tumor cells with the special up-regulation of WDR66 to create novel therapeutic opportunities for ESCC. It would be necessary to investigate the functional role of WDR66 by in vivo studies, and to analyze the WDR66 expression in a drug suppression manner for systemic delivery in ESCC.

7.3 WDR66 AFFECTS EMT OF ESCC

7.3.1 WDR66 MAY LEAD TO EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT)

According to a recent study comparing the risk of metastasis in ESCC and EAC, ESCC is characterized by a more aggressive behavior and tendency to metastasize at an early stage [39]. To form metastases, tumor cells have to undergo changes in order to be able to leave the primary tumor environment and to migrate to distant sites. Responsible for these morphological changes is a physiological process of epithelial-to-mesenchymal transition (EMT) which allows tumor cells to change their shape and polarity and to become motile. In the present study, over-expression of VIM and suppression of OCLN, both characteristics of EMT, were found in ESCC tissue when compared to normal esophagus tissue.

VIM is an important mesenchymal marker and plays a central role for reversible trans-differentiation [40]. Tumor cells undergoing EMT have an increased ability for detachment from the main tumor bulk. In line with this, the level of expression of VIM plays a predominant role in the changes in shape, adhesion, and motility that occur during the EMT [41]. EMT is also accompanied by a switch from keratin to VIM expression [42].

One of the important issues in tumor developing metastasis is the reduction of tight junctions. Herein, we found a decreased expression of OCLN in ESCC, an important tight junction protein. OCLN is the first discovered constituent of the epithelial tight junction and its role as a tumor suppressor has begun to emerge recently. Down-regulation of OCLN has been reported in various cancers [43]. A recent study showed that low expression of the tight junction protein claudin-4 is associated with poor prognosis in ESCC [44]. This is also in agreement with the results of our microarray study, which showed that claudin-4 was significantly less expressed in ESCC and also markedly over-expressed after WDR66 knockdown (data not shown).

Our experiments revealed a strong association of WDR66 expression with VIM and OCLN. Thus, we hypothesize that the elevated expression of WDR66 in ESCC may promote EMT through an up-regulation of VIM expression and a down-regulation of OCLN and cohesion

of the tumor tissue. We sought to determine if knockdown of WDR66 would alter the expression of VIM and OCLN, and lead to less invasiveness of tumor cells. We approached this by measuring their motility and apoptotic characteristics in the ESCC cell line.

7.3.2 REVERSING EMT BY WDR66 KNOCKDOWN

Due to the high over-expression of WDR66 in the majority of clinical ESCC samples, we were interested in modeling the role of this gene in the KYSE520 cell line (Human Japanese esophagus carcinoma squamous epithelial cell line) which also has a high expression of the WDR66 gene. The functional effects of VIM and OCLN in WDR66-knockdown KYSE520 cells were analyzed with regard to the gene expression level by qPCR and with regard to the protein level by western blotting. Gene expression level of VIM was significantly down-regulated whereas OCLN expression was significantly higher in cells treated with WDR66 siRNA (Figure 19). Detection of VIM and OCLN protein level by immunoblotting showed similar results, VIM expression was significantly down-regulated, while OCLN was significantly higher expressed in cells treated with WDR66 siRNA (Figure 20).

Having demonstrated an effect of WDR66 expression on VIM and OCLN in KYSE520 cells lines, we further investigated whether WDR66 might affect tumor cell motility. Cancer cells undergoing EMT can possess a migratory potential. Recent reports highlighted the importance of individual cell movement in cancer metastasis [45].

For KYSE520 cells treated with siWDR66, we proceeded to examine cell motility capacity of these cells compared to the cells transfected with control siRNA. We observed a significant suppression of cell growth in WDR66 knockdown cells. In order to visualize the involvement of WDR66 in cell migration and proliferation, we carried out wound healing assays with KYSE520 cells with WDR66 knockdown. These studies showed similar results, namely that cells transfected with siWDR66 significantly decreased wound closure and decreased cell motility. Although we examined the ability of WDR66 on KYSE520 cell motility, it must be noted that tumor cells are asked to migrate across a rigid and planar

substrate, which hardly resembles an in vivo situation, where cancer cells migrate into the extracellular matrix.

Together, the data presented by us create a strong link between the WDR66 gene and the EMT phenotype and show their importance in tumor progression.

7.3.3 WDR66-KNOCKDOWN INCREASES DRUG-MEDIATED CYTOTOXICITY TOWARD ESCC

Drug resistance has been a major problem in the fight against cancer and many chemotherapeutic drugs have been affected. The induction of EMT not only promotes tumor cell invasion and metastasis, but also contributes to drug resistance [45]. It may therefore be plausible to reduce tumor progression and drug resistance by targeting the EMT phenotype.

To determine whether WDR66 may also potentiate the cytotoxicity of chemotherapy to ESCC, Staurosporine and 5-Fluorouracil, the first line of chemotherapy in cancer therapy, were chosen to investigate its effect on KYSE520 cells line before or after WDR66 gene knockdown. In the present work, we are the first to report that siRNA-mediated knockdown of WDR66 resulted in suppression of cell growth, reduced cancer cell motility and enhanced cancer cell apoptosis after treatments with Staurosporine and 5-Fluorouracil. This finding could therefore yield therapeutic potential in targeting WDR66. But further in-depth investigation is still needed.

7.3.4 CLINICAL-TRANSLATIONAL ADVANCES

While the ideas presented above sound promising, testing in a clinical setting is still questioned due to the limited number of study populations. We evaluated the WDRR66 gene expression in patients with ESCC. High expression of WDR66 RNA was associated with poor tumor differentiation and higher TNM stage. In addition, multivariate Cox regression analysis revealed that WDR66 expression remained an independent prognostic factor. Most importantly, high expression of WDR66 RNA was found to be a significant

prognostic factor with regard to cancer-related survival. Our data imply a potential future benefit from the WDRR66-targeted therapy.

7.4 WDR66 AND OTHER NOVEL BIOMARKERS OF ESCC

In elucidating the biology of ESCC and the investigation of appropriate biomarkers relevant to the potential clinical use, here we compared the specificity of WDR66 with other biomarkers.

The serine/threonine kinase UNC-51-like kinase 1 (ULK1), which plays an essential role in autophagosome formation, represented a novel and clinically useful biomarker for ESCC patients and played an important role during the progression of ESCC [46]. Jiang et al. investigated the protein and mRNA levels of ULK1 in normal esophageal epithelial cells, ESCC cell lines, paired ESCC lesions and the adjacent noncancerous tissues using western blot and real-time RT-PCR. The result showed that only the ULK1 protein level was up-regulated in ESCC samples compared with normal esophageal cells and tissues. Compared with 96% of ESCC patients have enhanced expression of WDR66, only a total of 70.2% ESCC specimens showed intensive expression of ULK1. Functional studies showed that suppression of ULK1 expression in ESCC cell lines by specific small interfering RNA resulted in inhibition of cell proliferation and induction of apoptosis under starvation conditions which may probably be due to the importance of ULK1 in autophagy.

Tyrosine-protein phosphatase non-receptor type 12 (PTPN12) is considered to be a tumor suppressor and high expression of PTPN12 is associated with favorable disease-free survival and overall survival in ESCC patients [47]. By using Western blot in 20 pairs of surgically resected esophageal tissues, the higher PTPN12 expression was found in normal paracancerous esophageal tissues than in ESCC tissues. By immunohistochemical analysis, high and low expression of PTPN12 was found in 62.1% and 37.9% of ESCCs, respectively. The authors believe that the heterogeneity of biomarkers and low statistical power of single database may lead to this failure to show any significant correlation between PTPN12 expression and patient disease characteristics.

Galectin-7 is another potential biomarker for cancers including ESCC [48]. Up-regulation of galectin-7 was further confirmed by western blotting and immunohistochemistry. Furthermore, immunohistochemical staining of galectin-7 was performed on a tissue microarray containing ESCC samples and normal esophageal epithelial tissues samples. The expression levels of galectin-7 were markedly higher in the ESCC samples than in the normal esophageal epithelial tissues. But its prognostic value is still under investigation.

Compared with these biomarkers of ESCC, WDR66 was specifically highly expressed in ESCC patients and showed a strong correlation with the overall survival rate. The functional role of WDR66 in ESCC may be explained by the elevated expression of WDR66 in ESCC may promote EMT through an up-regulation of VIM expression and a down-regulation of OCLN. When knockdown of WDR66 can alter the expression of VIM and OCLN, and make tumor cells less invasive in vitro by measuring their motility and apoptotic characteristics. In spite of the small number of patients sampled, we believe that our finding is promising and helps us to obtain a clearer picture of WDR66 function in ESCC biology.

7.5 OUTLOOK

In summary, we have identified WDR66 as a potential novel prognostic marker and promising target for ESCC. This result is based on our observations that (1) WDR66 is specifically highly expressed in esophageal squamous cell carcinoma and that high WDR66 expression correlates with poor overall survival, (2) WDR66 regulates vimentin and occludin expression and plays a crucial role for EMT, and (3) knockdown of WDR66 suppresses cell growth and motility and decreases cell viability of ESCC cells. Therefore, we propose that WDR66 plays a major role in ESCC biology. Our functional data furthermore warrants further investigation of selective targeting of WDR66 as a novel drug target for ESCC treatment.

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9. STATEMENT
Affidavit

“I, [Chenming Ma] certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Functional analysis of the WDR66 gene] I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interests in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date 29.07.2013 Signature Chenming Ma

Declaration of any eventual publications

[Chenming Ma] had the following share in the following publications:

Publication 1: [Chenming Ma, Qing Wang, Yicun Man, Wolfgang Kemmner.], [Cardiovascular medications in angiogenesis-How to avoid the sting in the tail], [Int J Cancer], [2012]

Contribution in detail: Ma have made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the manuscript and revising it critically for important intellectual content.

Publication 2: [Xu Qian, Steffen Wagner, Chenming Ma, Jens P. Klussmann, Michael Hummel, Andreas M. Kaufmann, Andreas E. Albers.], [ALDH1-positive cancer stem-like cells are enriched in nodal metastases of oropharyngeal squamous cell carcinoma independent of HPV status], [Oncol Rep], [2013]

Contribution in detail: Ma has been involved in data analysis and interpretation of data.

Publication 3: [Qing Wang, Chenming Ma, Wolfgang Kemmner.], [Wdr66 is a novel marker for risk stratification and involved in epithelial-mesenchymal transition of esophageal squamous cell carcinoma], [BMC Cancer], [2013]

Contribution in detail: Ma has been involved in data acquisition, data analysis and interpretation of data, drafting the manuscript and revising it critically for important intellectual content. Ma is equal first author.

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

_____

10. CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

11. PUBLICATIONS

1.Wenjing Li, Tengke Wang, Chenming Ma, et al. Calcitonin gene-related peptide inhibits interleukin-1{beta}-induced endogenous monocyte chemoattractant protein-1 secretion in type II alveolar epithelial cells. Am J Physiol Cell Physiol. 2006;291:456-465.

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