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DISSERTATION

The role of Notch signaling pathway in cisplatin sensitivity and radiation sensitivity in head and neck squamous cell carcinoma

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Abstrakt

Die Behandlung von Kopf-Hals-Plattenepithelkarzinomen (HNSCC) ist oft komplex und beinhaltet ein multimodales Management, welches die Chirurgie, Strahlentherapie und systemische Chemotherapie einschliesst. Für Patienten mit einem lokal fortgeschrittenem HNSCC kommt eine Cisplatin-basierte Chemotherapie in Kombination mit einer Strahlentherapie als Standard-Behandlung zur Anwendung. Obwohl sich die Prognose durch die Hinzunahme von Cisplatin zur Strahlentherapie verbessert hat, kommt es nach wie vor auch nach dieser intensivierten Therapie zu Rezidiven, möglicherweise durch die Anwesenheit von resistenten Tumorzell-Subpopulationen in diesen Fällen. Es ist daher unerlässlich, neue Strategien zur Verbesserung der Antitumorwirkung von Cisplatin zu entwickeln.

Eine vorangegangene Studie unserer Arbeitsgruppe hat gezeigt, dass der Notch-Signalweg an Cisplatin oder Radio-Resistenz beteiligt sein könnte. Patienten mit Notch1-Mutationen reagierten signifikant empfindlicher auf eine Cisplatin-basierte Chemo-Strahlentherapie, verglichen mit Patienten ohne Notch1-Mutationen. Die genauen molekularen Ursachen für die unterschiedliche Therapiewirksamkeit in Abhängigkeit des Notch1-Mutationsstatus sind jedoch unbekannt. In der vorliegenden Arbeit wurde daher die Wirkung der Hinunterregulation von Notch auf die Sensitivität von HNSCC-Zellen gegenüber Cisplatin oder Strahlentherapie untersucht. Die humanen HNSCC-Zelllinien FaDu und UD-SCC-4 und daraus abgeleitete Zelllinien mit Resistenz gegenüber Cisplatin (FaDu $_{CDDP-R}$ und UD-SCC-4 $_{CDDP-R}$) wurden dafür verwendet. Zur Hemmung des Notch-Signalsweges wurden diese Zellen mit kleiner interferierender RNA (siRNA) für Notch transfiziert. Alternativ wurden die Krebszellen mit dem γ-Sekretase-Inhibitor (GSI IX) behandelt, was die Bildung von aktivem Notch-Protein hemmt. Der Einfluss der Hemmung des Notch Signalweges auf die Viabilität der Krebszellen wurde durch den MTT-Assay bestimmt. Die Notch-Expression in den HNSCC-Zelllinien wurde nach Transfektion mit siRNA unter Verwendung von Real-time-PCR und Western-Blot-Analyse gemessen. Die Expression der Zielgene im Notch-Signalweg (*HES1* und *HEY1*) wurden ebenfalls mittels Real-time-PCR gemessen.

Die Untersuchungen ergaben, dass sowohl das unprozessierte Notch1 (F-Notch1) als auch die prozessierte aktivierte Form von Notch1 (C-Notch1) in den Cisplatin-resistenten HNSCC-Zelllinien hinaufreguliert war. Die Hemmung der Expression von Notch1 und Notch3 durch siRNA-Transfektion änderte jedoch die Empfindlichkeit gegenüber Cisplatin in HNSCC-Zellen nicht ($p > 0,05$). Wenn Zellen 24 Stunden vor Cisplatin mit GSI IX behandelt wurden, wurde die Empfindlichkeit gegenüber Cisplatin signifikant verringert ($p < 0,05$). Darüber hinaus wurde ein signifikanter Unterschied der aktivierten Caspase-3 zwischen Cisplatin-behandelten Zellen und

Cisplatin in Kombination mit GSI-IX-behandelten Zellen beobachtet. Der Notch1 Signalweg scheint daher eine entscheidende Rolle bei der Cisplatin-Sensitivität in HNSCC-Zellen zu spielen und seine pharmakologische Hemmung mittels GSIs eine negativen Auswirkung auf die Wirksamkeit der Cisplatin-Therapie zu haben. Dieser Befund ist wichtig, da der Einsatz von GSIs zur Chemosensibiliserung bereits in klinischen Phase I Studien bei Patienten mit Kopf-Hals-Karzinom getestet wird. Eine weitere Überprüfung der Rolle der GSIs bei HNSCC sollte daher erfolgen.

Abstract

The treatment of head and neck squamous cell carcinoma (HNSCC) involves multimodality management, including surgery, radiotherapy and systemic chemotherapy. Cisplatin-based concurrent chemo-radiotherapy is now considered to be the standard treatment for patients with locally advanced unresectable HNSCC. However, though addition of cisplatin to radiotherapy improved outcome, the number of patients presenting with recurrences is still high, probably due to the presence of cisplatin resistant tumor cell subclones in these cases. It is imperative to develop new strategies to improve the antitumor effect of cisplatin.

The results from a previous study of our group were suggestive that the Notch signalling pathway might be involved in cisplatin or radio-resistance. Tumors with Notch1 mutations were significantly more sensitive to cisplatin based chemo-radiotherapy, compared to tumors without Notch1 mutations. However, the molecular basis for the different treatment efficacy depending on the NOTCH mutational status remains unresolved. In the current project, the effect of *NOTCH* gene silencing on the cisplatin or radiotherapy sensitivity of HNSCC cells was therefore investigated. The human HNSCC cell lines (FaDu and UD-SCC-4) and cisplatin-resistance cell lines derived thereof (FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$) were used in this project. The role of Notch1 in cisplatin resistance was studied by transfecting cells with small interfering RNA (siRNA) targeting Notch. Additionally, cancer cells were treated with γ-secretase inhibitor (GSI IX), which inhibits Notch1 activation by proteolytic cleavage. Relative viability of cancer cells was determined by the MTT assay. Notch expression in the HNSCC cell lines following transfection with siRNA was determined using real-time RT-PCR and western blot analysis. The target genes in the Notch signaling pathway (Hes1 and Hey1) were measured by real-time RT-PCR.

Both full length Notch1 (F-Notch1) and activated cleaved Notch1 (C-Notch1) were found to be up-regulated in the cisplatin resistant HNSCC cell lines. However, silencing of Notch1 and Notch3 by siRNA transfection did not change the sensitivity of HNSCC cells to cisplatin ($p>0.05$). When cells were treated with GSI IX twenty-four hours before addition of cisplatin, the sensitivity to cisplatin was significantly decreased in these four cell lines ($p<0,05$). Furthermore, a significant difference of activated caspase-3 was observed between cells treated with cisplatin alone and its combination with GSI IX. Thus, the Notch1 signalling pathway seems to play a critical role in sensitivity of HNSCC cells to cisplatin. It is important to notice that GSIs might decrease tumor cell sensitivity to cisplatin as they have already been used in HNSCC patients in phase I clinical

trials as chemotherapy sensitizer. Thus, the molecular basis for the unexpected reduction of cisplatin sensitivity of HNSCC cells after GSI-treatment should be further explored in future studies.

1. Introduction

1.1 Head and neck cancer

Head and neck cancer (HNC) is a very heterogeneous group of cancers, which is not referred to a single site or single histological type. The ICD-10 classification for HNC includes tumors of the larynx, hypopharynx, oropharynx and oral cavity. Around 90-95% of head and neck cancers are squamous cell carcinoma (HNSCC) [1]. In Europe, HNC accounts for approximately 3.2% of the estimated 3 439 000 new cases of cancer and roughly around 3.6% of 1 754,000 cancer-related deaths for the year 2012 [2]. According to a recent survey in China (Data from 22 registries, representing 6.5% of the whole population in China), it was estimated that 74 300 people (54 800 men and 19 500 women) were newly diagnosed with HNC and 36 600 deaths (27 900 men and 8 700 women) occurred in 2015, accounting for 2.0% of all cancer incidence and 1.0% of all cancer deaths [3].

Despite of recent advances in surgical and oncological practice, overall survival rates for HNSCC patients have remained relative poor [4]. About two-thirds of HNSCC patients still present with locally advanced disease, commonly involving regional lymph nodes, due to late diagnosis. Though survival rates improved slightly over the last few decades, 30-60% of patients still develop local recurrences, and 20% develop distant metastases even after radical treatment [5]. For these patients, the 5-year overall survival rates are less than 50% with severely reduced quality of life [4].

1.1.1 Etiology of HNSCC

There is no single etiological risk factor which has been attributable to developing HNSCC. Tobacco and alcohol have been identified as the two main established risk factors for development of at least 70% of HNSCC [6-8]. A dose-effect relationship between duration of smoking or alcohol and increased risk of cancer and risk reduction after cessation of smoking or alcohol were observed in several studies [9-11]. Although smoking and alcohol are the predominant causes of the most HNSCC, other factors such as diet are relevant in some patients [12]. Moreover, genetic

and epigenetic factors which may contribute to the disease are under investigation [13-15]. Additionally, recent laboratory and epidemiological evidences demonstrated that the human papilloma virus (HPV) emerged as a risk factor for HNSCC, especially in young adults [16]. Interestingly, the HPV status has a positive prognostic value in HNSCC, as patients with HPV-positive HNSCC have better prognosis compared to patients with HPV-negative HNSCC [17]. One possible reason is that patients with HPV-positive HNSCC have a better therapeutic response to chemo-radiotherapy [18].

1.1.2 Treatment of HNSCC

The overall purpose of treatment in HNSCC is to largely remove the cancer load, prevent subsequent recurrence or metastasis, and ideally preserve the most important functions including swallowing, speaking and breathing. The mainstays of treatment are surgery, radiotherapy, chemotherapy, or a combination of these treatments. Generally, for early stage disease without regional lymph node metastasis, surgical excision or curative radiotherapy are used. For locally advanced HNSCC, the treatment is usually a combination of surgery or radiotherapy and adjuvant chemotherapy [19]. However, nearly half of patients will develop local-regional recurrence or distant organ metastasis [19, 20]. The aim of treatment for these patients is to prolong overall survival and achieve the palliation of symptoms. Salvage excision [21], re-irradiation [22, 23], palliative chemotherapy [24], targeted therapy [25] or immune therapy [26] can be used in these patients.

1.1.3 Chemotherapy in HNSCC

There is no evidence to support the use of chemotherapy alone in the treatment of head and neck cancer, except in palliative settings. However, chemotherapy plays an important role in the management of HNSCC. Adjuvant chemotherapy to radical surgery or radiotherapy could improve local-regional disease control rate, decrease the incidence of distant organ metastasis [27], and increase the incidence of important organ preservation [28]. One large meta-analysis, published in 2009, included 93 randomized trials and 17 346 patients [29]. This study demonstrated that the benefits of addition of chemotherapy to radiotherapy in locally advanced HNSCC were: 1) improved overall survival by 4.5% at 5 years; 2) improved prognosis when concomitant rather than induction chemotherapy was used; 3) increased overall survival by 6.5% at 5 years when concomitant chemotherapy was used; 4) improved outcome if cisplatin was used in chemotherapy regimens, either cisplatin alone or combined with any other reagent. In another large meta-analysis, the magnitude of the benefit according to tumor site in HNSCC was evaluated [30]. The

5-year absolute benefits associated with the concomitant chemotherapy are 8.9%, 8.1%, 5.4% and 4% for oral cavity, oropharynx, larynx and hypopharynx tumors, respectively. Cisplatin or carboplatin alone, or combined with 5-fluorouracil (5-FU) gave a benefit of the similar order of magnitude in all subtypes of HNSCC. Compared with carboplatin, patients with cisplatin-based chemotherapy can achieve a higher overall survival rate and associate with fewer hematological toxicities [31]. As a result, cisplatin with or without 5-FU concurrently delivered with conventional daily fractionated radiation therapy is the standard chemo-radiation regimen in clinical practice [32].

1.1.4 Cisplatin based chemotherapy in HNSCC

Cisplatin (cis-[Pt Cl_2 $(NH_3)_2$]) was discovered more than one hundred years ago [33, 34]. In 1978, it was approved for use in testicular and ovarian cancers by the U.S. Food and Drug Administration. Now, it has maintained widespread clinical use in solid tumors, with a broad spectrum of clinical activity in numerous malignancies, including HNSCC. Different chemical derivatives of cisplatin have been evaluated as potential chemotherapeutic agents, but cisplatin is the most active and has led to the cure of over 90% of testicular cancer cases [35]. It plays a vital role in the treatment of cancers such as ovarian [36], HNSCC, bladder cancer [37], cervical cancer [38], lung cancer [39], esophageal cancer [40], as well as several others [41]. Cisplatin is generally believed to exert its anticancer effects by interfering with DNA repair mechanisms, causing DNA damage and subsequently inducing apoptosis [42].This occurs when cisplatin reacts with the N-7 atoms of the purine bases, forming 1, 2-intrastrand cross-links as major adducts. The minor adducts are interstrand crosslinks or 1, 3-intrastrand crosslinks. These DNA adducts cause significant distortion of the DNA that can be recognized by DNA binding proteins. These proteins are involved in initiating DNA damage repair, inhibiting essential transcription or triggering signals for programmed cell death (apoptosis) [43]. The cytotoxicity of cisplatin is mediated by HMG1 (High mobility group 1) and HMG2 proteins, a group of platinum-damage recognition proteins binding selectively to cisplatin-DNA adducts [44]. The high binding affinity of these proteins have been postulated to block DNA damage repair and DNA synthesis resulting in activation of apoptotic processes [45]. The loss of the HMG protein has been shown to result in increased nucleotide excision repair of cisplatin-DNA adducts and decreased cytotoxicity of cisplatin [45]. Furthermore, cisplatin could activate p38 MAPK signaling pathway which has been implicated in the better response to cancer therapy [46]. Specific p38 MAPK inhibitor could significantly decrease the cisplatin sensitivity [46].

In clinical settings, patients usually have a good response to cisplatin-based chemotherapy at the beginning of treatment but present with relapse, due to the development of cisplatin resistance [47]. The precise molecular mechanisms of cisplatin resistance have not yet been elucidated. The cellular mechanisms of cisplatin resistance have been identified as decreased intracellular concentration as a result of decreased drug uptake, increased efflux or increased inactivation by sulfhydryl molecules such as glutathione [48]. This resistance can also result from other factors, including increased levels of DNA damage repair (nucleotide excision repair and mismatch repair), changes in DNA-methylation status, alterations of membrane protein trafficking as a result of defective organization and distribution of the cytoskeleton, overexpression of chaperones, up-regulated or down-regulated expression of transcription factors, inactivation of the apoptosis pathway and activation of the epithelial mesenchymal transition (EMT) pathway [49]. Furthermore, investigations of our group showed that there is a selection towards a resistant subpopulation indicating the presence of resistant cells from the beginning rather than acquiring resistance by therapy-induced mutations (Niehr et al, unpublished).

1.1.5 Cisplatin resistance in HNSCC

Despite recent advances in targeted therapy, cisplatin remains the standard first-line chemotherapeutic agent for treatment of HNSCC. However, over the last three decades, the prognosis in HNSCC has not changed substantially, a result of primary or acquired resistance of cancer cells to cisplatin. Therefore, one possible solution is to avoid or overcome cisplatin resistance in tumor cells.

Over the last decade, a huge number of studies have been initiated to overcome this clinical problem of cisplatin resistance. These studies can be classified into three groups [50]: 1) genomic, transcriptomic, methylomic and proteomic approaches, in preclinical models or patients' materials; 2) large-scale silencing approaches and functional screenings, determining whether the genetic or pharmacological inhibition of specific proteins alters cisplatin sensitivity, in preclinical models; 3) multiplex genotyping studies, assessing, whether single-nucleotide polymorphisms (SNPs) are associated with increased or reduced cisplatin sensitivity in clinical settings in a high-throughput manner. Although these studies provided more precise insights into the molecular mechanisms that might be responsible for cisplatin resistance, there were few clinically applicable strategies for solving this problem [50]. Even, the use of cisplatin in combination with targeted drugs, such as cetuximab (a monoclonal antibody that blocks ligand binding to the epidermal growth factor receptor [EGFR]), erlotinib (a pharmacological inhibitor of the tyrosine kinase

activity of EGFR) and bevacizumab (a vascular endothelial growth factor-blocking antibody), failed to increase cisplatin sensitivity in phase II-III randomized clinical trials [51-53].

1.1.6 Radiotherapy in HNSCC

Since X-rays were discovered in 1895, the first cured cancer patient by irradiation was diagnosed with nasal basal cell carcinoma. The high-energy photons initially interact with living tissue and generate high-energy electrons, which create secondary ionization events. It is wildly accepted that nuclear DNA is the critical target for radiation induced cell death [54]. When ionization is absorbed by living tissues, the interaction of radiation and tissues results in the generation of free radicals, particularly the hydroxyl radical (OH^-). The hydroxyl radical can cause a break of DNA helix, especially fatal double-strand breaks (DSB). The subsequence of radiation will not only depend on the dose of irradiation given, but also on the ability for detecting and repairing the damages in the cell. Until now, radiotherapy is used for treatment of nearly 75% of all HNSCC patients, with either curative or palliative intent. In patients with early stage disease, radiotherapy could offer outcome comparable to that achieved by surgery but with lower morbidity [55]. In advanced stage tumors, adjuvant radiotherapy increased the absolute 5-year cancer specific survival and overall survival by 10%, compared with surgery alone, according to a report from the Surveillance, Epidemiology, and End Results (SEER) database [56]. Furthermore, when patients develop recurrence or metastasis, radiotherapy could be used as salvage or palliation, with acceptable toxicities as well [57].

Despite the emergence of new approaches and treatment modalities, loco-regional recurrence is considered to be the main cause of death in patients with HNSCC. One of the possible reasons is that cancer cells develop resistance to radiotherapy. Therefore, overcoming radio-resistance may provide the opportunity to cure HNSCC.

1.1.7 Cisplatin based concurrent chemo-radiotherapy in HNSCC

Concurrent cisplatin-based chemo-radiotherapy is now considered the standard first-line treatment for patients with locally advanced HNSCC [29]. Cisplatin is a potent radiosensitizer which could improve the outcome of radiotherapy [58]. A prior meta-analysis investigating various chemo-radiotherapy regimens indicated that cisplatin-containing regimens may provide a survival advantage compared with non-cisplatin-containing regimens [59]. Furthermore, cisplatin is recommended to be used as a combined modality approach integrating irradiation to avoid the cross-resistance between chemotherapy and radiotherapy [60]. Therefore, the current most widely used standard regimen is 100 mg/m^2 cisplatin every 3 weeks, combined with 66-70 Gy radiation

delivered in 1.8-2.0 Gy daily fractions. However, this treatment regimen causes very severe toxic effects, such as nephro-, oto- and neurotoxic effects, nausea and severe mucositis. Most patients could not tolerate this regimen and only patients with a good performance status and normal kidney function are suitable for this kind of therapy [61]. Additionally, even with this regimen, the 3-year overall survival rate was only 37% according to a large series clinical report [32]. To limit toxic effects and improve patients' outcome, alternative administration schedules are also being used, such as once-weekly administration of smaller cisplatin doses [62], intra-arterial administration of cisplatin [63] and as well as daily administration of cisplatin [64] at doses of 5-8 mg/m^2. Theoretically, these methods have the potential to optimize its radiosensitizing properties, but randomized phase III clinical trials are needed to support this hypothesis. Thus, it is imperative to develop new strategies to improve the antitumor effect of cisplatin based concurrent chemo-radiotherapy. An ideal strategy would be the identification of a nontoxic agent which act synergistically with cisplatin based concurrent chemo-radiotherapy triggering the cell death preferentially in tumor cells. Recently, investigations of our group revealed that detection of *NOTCH1* mutations in tumor tissue was significantly correlated with improved survival after cisplatin based concurrent chemo-radiotherapy, compared to patients with wild type Notch1 [65]. Although these results point to a role of the Notch signaling pathway in cisplatin or radio-resistance in HNSCC, the molecular basis for this association remains unclear.

1.2 NOTCH signal pathway in HNSCC

Notch signaling is a highly evolutionally conserved pathway that plays a pivotal role in metazoan development, tissue renewal, and cell-fate determination of progenitors [66]. In mammals, there are four Notch receptors (Notch 1-4) and five known Notch ligands (Jagged [JAG] 1-2, Delta-like [DLL] 1, 3 and 4), all of which are type I transmembrane proteins. As shown in Figure 1.1, Notch protein precursors are synthesized in the endoplasmic reticulum and mature in the Golgi apparatus where they are cleaved by furin-like convertase (S1 cleavage). Following S1 cleavage, the mature Notch receptor is activated by the interaction of the Notch receptor with its ligands between two neighboring cells. Upon ligand binding, the interaction induces a conformational change, exposing the S2 cleavage site to an ADAM-family metalloprotease. S2 cleavage, also named as ADAM-dependent cleavage, generates the membrane-anchored Notch extracellular truncation fragment (NEXT), an extremely short-lived substrate for the γ-secretase complex. Following S2 cleavage, γ-Secretase complex cleaves the Notch transmembrane domain in NEXT at S3 site to release the active Notch intracellular domain (NICD). After this final cleavage, the cleaved product, NICD translocated into the nucleus where it associates with the

nuclear DNA-binding proteins, (CSL/RBPjk complex), and recruits co-activator protein mastermind-like 1 (MAML1) to modulate target gene expressions. The most prominent target genes of Notch proteins include *hairy enhancer of split* genes (*HES*) and *hairy/enhancer-of-split related with YRPW motif protein* genes (*HEY*) [67].

The multi-subunit protease γ-secretase is one of the key enzymes during the Notch signal activation. Indeed, γ-secretase inhibitors (GSIs) block the generation of Notch intracytoplasmic domain and with this the activation Notch signaling pathway. Subsequently, blockade of Notch suppresses abnormal cell differentiation. Although γ-secretase cleaves within the transmembrane domains of over 100 type 1 membrane proteins [68], development of GSIs for most cancers has been primarily focusing on the inhibition of Notch1 cleavage [69, 70]. GSI IX (DAPT) and GSI XII were two of the most common used GSIs for Notch inhibition in biological experiments [69, 71, 72].

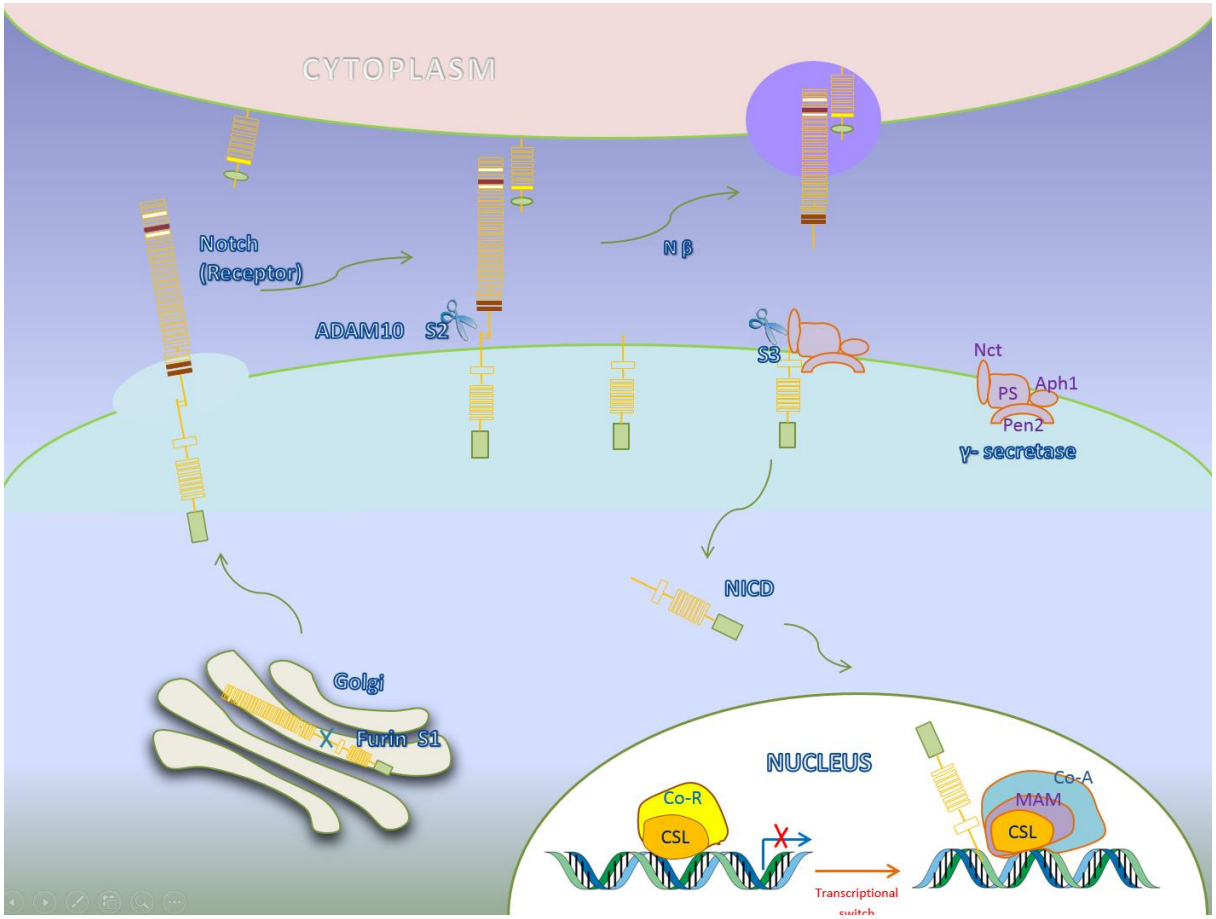


Figure 1.1: Overview of Notch proteolysis: A schematic overview of the Notch signaling pathway presenting the proteolytic procedure.

1.2.1 *NOTCH1* mutation in HNSCC

Next-generation sequencing (NGS) has identified *NOTCH1* as one of the most frequently mutated gene (Next to *TP53*), with alterations present in approximately 15-20% of HNSCC patients [73-75]. Among the identified *NOTCH1* mutations, considerable alterations were loss-of-function mutations [74]. These evidences suggested that *NOTCH1* function as a tumor suppressor role in HNSCC.

To further understand the *NOTCH1* mutations in HNSCC, public available *NOTCH1* mutations data were summarized by Zhang et al [76]. As shown in Table 1.1, the majority of these *NOTCH1* mutations were mainly located at the epidermal growth factor (EGF) ligand-binding region, negative regulatory region (NRR) domain and ankyrin repeats (ANK) domain. The missense mutations in EGF region likely disrupt Notch1 folding and affect Notch ligand binding [77]. The mutations in NRR domain are predicted to cause cysteine crosslink between molecules and disrupt Notch folding. The mutations in ANK domain probably affect CSL binding and disrupt Notch folding likewise. It has been well known that the *EGFR* mutation rate is highly different between Eastern Asian population and Caucasian population. Interestingly, the *NOTCH1* mutations rate is also variable in different ethnicities. In Chinese, 43% oral squamous cell carcinoma (OSCC) patients carry *NOTCH1* mutation [78], compared to less than 20% in HNSCC patients in Caucasian population. Furthermore, a more complicated *NOTCH1* mutation status was observed in Chinese patients, due to the different inherited genomic background. The *NOTCH1* HD domain mutation was another common domain, which was not often observed in Caucasian solid tumor patients [78].

Table 1.1 *NOTCH1* mutations involved in HNSCC and their predicted functional consequences [76]

NOTCH1 mutations	Position	Predicted functional consequences
P422S	EGF11	Disrupting ligand binding
C429Y	EGF11	Disrupting Notch folding or causing cysteine crosslink
C440F/R	EGF11	Disrupting Notch folding or causing cysteine crosslink
N454T	EGF12	Disrupting calcium binding
E455K	EGF12	Disrupting calcium binding
C461Y	EGF12	Disrupting Notch folding or causing cysteine crosslink
A465T	EGF12	Disrupting ligand binding
C478S/Y	EGF12	Disrupting Notch folding or causing cysteine crosslink

G481S	EGF12	Benign
G481C	EGF12	Causing cysteine crosslink
G484V	EGF12	Benign
C1085Y/W	EGF28	Disrupting Notch folding or causing cysteine crosslink
R1520H	NRR	Disrupting calcium binding or Notch folding
N1875K	ANK	Benign
T1996M	ANK	Interfering with transcription factor binding
A2023T	ANK	Disrupting Notch folding or changing ANK conformation
P2064L	ANK	Disrupting Notch folding or changing ANK conformation

EGF, epidermal growth factor; ANK, ankyrin repeats; NRR, negative regulatory region.

1.2.2 Notch1 as a tumor suppressor in HNSCC

As mentioned above, there is evidence of a tumor suppressor role of Notch1 in HNSCC. However, to date, functional studies clarifying the tumor suppressive role of Notch1 in HNSCC are limited [79]. The biological significance of Notch1 as tumor suppressor in HNSCC has been already investigated in *in-vitro* studies. A summary of these studies is shown in Table 1.2. Constitutively over-expressed active Notch1 significantly suppressed tumor cell proliferation, via induced G_0-G_1 cell cycle arrest and decreased apoptosis [80]. Consistently, tumor cell growth was obviously inhibited by re-activation of NICD1 using a retroviral construct in OSCC cell lines, which harbored missense or truncating *NOTCH1* mutations [81]. One possible mechanism is that activation of Notch1 signaling could lead to dramatic increase in p21 and p53 expression with decreases in Bcl-2 (B-cell lymphocytic-leukemia proto-oncogene 2) and β-catenin expression, which may participate in the induction of apoptosis and cell cycle arrest [80]. Furthermore, several reports investigated protein levels of Notch1 between primary HNSCC tumor tissues and corresponding normal epithelium and found decreased Notch1 expression in tumor cells [79, 82].

Table 1.2 Summary of *in vitro* studies demonstrating that Notch1 can be a suppressor in HNSCC

Ref	Year	Result	Implication
[80]	2006	Constitutively over-expressed active Notch1 significantly suppressed tumor cell proliferation.	Cancer cell proliferation.
[81]	2013	Overexpression of active Notch1 significantly suppressed tumor cell proliferation.	Cancer cell proliferation.
[82]	2012	Notch1 knockdown cells formed a dysplastic stratified epithelium mimicking a precancerous lesion.	Cancer cell differentiation.

1.2.3 Notch1 as an oncogene in HNSCC

The first evidence for potential oncogenic role of the Notch signaling pathway was coming from studies reporting overexpression of Notch1 and Notch2 proteins in tumor tissues of HNSCC patients [83]. Recently, mRNA levels of several key molecules, such as Notch1, Notch2, Jag1,

Hes1 and Hey1, were found to be increased in OSCC, compared with surrounding normal tissues [84, 85]. In clinical studies, the expression of Notch1 protein was found to be positively related with cervical lymph node metastasis [86, 87], tumor invasion and micro-vessel density [88]. Furthermore, in a clinic-pathological analysis [89], Notch1 expression correlated with both the T-stage and the clinical stage. Notch1 and its NICD1 were found to be characteristically localized at the invasive tumor front, which means Notch1 plays an important role in tumor cell invasion and metastasis. Additionally, high expression of Notch1, detected by immunohistochemistry (IHC), was positively associated with cisplatin sensitivity resistance in cells isolated from HNSCC patients [90]. Elevated Notch1 expression was also related with short disease-specific survival time and overall survival time [91].

The biological significance of Notch1 as an oncogene in HNSCC has been already investigated in *in-vitro* studies. A summary of these studies is shown in Table 2. Knockdown of Notch1 by siRNA transfection or inhibition of activated Notch by treating cells with γ-secretase inhibitor [89, 92] significantly reduced cell proliferation and decreased the ability of invasion. The activation of Notch1 signaling pathway, either by induction of its ligand or treatment with tumor necrosis factor alpha (TNFα), led to maintenance of stem-like cells [85, 87, 91].

Table 1.3 Summary of *in vitro* studies demonstrating that Notch1 can be oncogenic in HNSCC

Ref	Year	Result	Implication
[85]	2012	Notch pathway inhibition using a gamma-secretase inhibitor prevented tumor growth.	Cancer cell proliferation.
[87]	2016	HNSCC cell lines overexpressing Notch1 are enriched in stem cell markers and form spheroids. Knockdown of Notch1 inhibited spheroid forming capacity, transformation, survival and migration of the HNSCC cells.	Cancer stem cell, Migration.
[89]	2013	Knockdown of Notch1 or treatment with GSIs reduced cell proliferation and invasion.	Cancer cell proliferation, invasion.
[91]	2016	Constitutive activation of Notch1 increased the expression of stem cell markers such as Oct4, Sox2, and CD44.	Cancer stem cell.
[92]	2014	Knockdown of Notch1 or treatment with GSIs reduced cell proliferation.	Cancer cell proliferation.

1.3 Aims of the thesis

Cisplatin-based chemotherapy and radiotherapy play an important role in treatment of HNSCC. Radio- and chemo-resistance are the most common challenges in clinical settings. Although Notch1 has been studied in human cancer cells for almost two decades [93], there are still a lot of

open questions, especially about its role in HNSCC. The role of Notch signaling pathway in carcinogenesis is variable. As mentioned above, the Notch1 can act as tumor suppressor or oncogene, depending on tumor entity, tumor cellular context and tumor mutation. Furthermore, the relationship between Notch1 signaling pathway and radiochemo-resistance is not well elucidated. It is especially urgent to clarify the role of Notch1 and its family members in radiochemo-resistance and whether Notch1 acts as tumor suppressor or oncogene. Elucidating the function of the Notch signaling pathway in HNSCC cells may pave the way to refine clinical diagnostics, to develop promising new therapeutic strategies and to improve treatment selection by stratification of patients according to the mutational status or Notch1 expression pattern. Therefore, the aim of this thesis was to investigate the role of Notch signaling in treatment resistance of HNSCC cells. For that purpose, the expression of Notch1 in different resistance models in HNSCC cell lines was analyzed. In addition, it was investigated whether sensitivity of HNSCC cells to cisplatin or irradiation could be increased by specific inhibition of the Notch pathway, using either knockdown by Notch siRNA or treatment of cells with γ-secretase inhibitors.

2. Methodology

2.1.1 List of reagents

Reagent	Company	Catalogue Number	Volume
DMEM/F12(1:1)(1 X)	Gibco by life technologies	11330-032	500 ml
Fetal Calf Serum (FCS)	Lifetechnologies	10500-064	500 ml
PBS Dalbecco (w/o Ca2+, Mg2+)	Biochrom	L1825	500 ml
0.25% Trypsin EDTA	Sigma	T4049-500ML	500 ml
Pure water	Fresenius KABI	13HLP021	1000 ml
Ethanol (≥99.8%)	Roth	K928.4	5 L
NEAA 100×	Lifetechnologies	11140035	400 ml
Cisplatin	Sigma-Aldrich	721816	2 ml
γ-secretase inhibitor XII	Calbiochem	565773	5 mg
γ-secretase inhibitor IX	Selleck chemicals	S2215	5 mg
Dimethyl sulfoxide	Sigma-Aldrich	A994.1	100 ml
Notch1 siRNA	Dharmacon	L-007771-00-0005	5 nmol
Notch3 siRNA	Dharmacon	L-011093-00-0005	5 nmol
Negative control siRNA	Dharmacon	D-001810-01-05	5 nmol
Transfection Reagents	DharmaFECT 1	T-2001-02	750 µl
MTT reagents	Roth	4022.2	10 g
RIPA buffer	ThermoScientific	89901NA	250 ml
ß- Mercaptoethanol	Sigma	M3184-25	25 ml
Methanol	Roth	4627.4	1000 ml
Halt Protease & Phosphatase Inhibitor Cocktail	ThermoScientific	78440	1 ml
0.5 M EDTA solution	ThermoScientific	1861274	1 ml
BCA protein assay kit	Thermo scientific	23227	500 ml
TEMED	Roth	4627.4	100 ml
Rotiphorese Gel 30	Roth	3029.2	500 ml
Nonfat dry milk	Applichem	A0830-1000	1 kg

Tris-Base	Roth	5429.3	1 kg
Glycin	Sigma	G8898	1 kg
SDS	Roth	D-76185	1 kg
Sodium Chloride	Merck	7647-14-5	1 kg
Tween	Sigma	STBB6902V	500 ml
ECL plus Western Blotting Detection Reagents	Thermoscientific	32132	100 ml
Development Solution	Carestream	191875	500 ml
Fixing Solution	Carestream	191859	500 ml
LightCycler 480 Probes Master	Roth	04707516001	1 ml
Rnase Inhibitor 40U/µl	Thermoscientific	EO0382	4x2500U
Oligo dT Primer p(dT)15	Roth	10814270001	40 µg

2.1.2 List of buffers and kits

Name	**Components**
4x Lämmli-Buffer	24 ml 1M Tris-Base, 8 g SDS, 40 ml Glycerol, 10mg Bromphenol 28 ml dH_2O Freshly add 11.1 µl mercaptoethanol to 100 µl 4x Lämmli-Buffer, when perform western blot.
10x Electrophoresis Buffer	30.3g Tris-Base, 142.6g Glycin, 10g SDS, PH 8,3. Fill up to 1 L with dH_2O
1x Electrophoresis Buffer	100 ml 10x Electrophoresis Buffer 900 ml dH_2O
10x Transfer Buffer	58.1 g Tris-Base, 25.3 g Glycin. Fill up to 1Lwith dH_2O
1x Transfer Buffer	50 ml 10x Transfer Buffer, 100 ml Methanol Fill up to 500 ml with dH_2O
10x TBS	24.2 g Tris-Base, 80 g NaCl. Fill up to 1 L with dH_2O
1x TBS	100ml 10x-TBS

	900ml dH_2O
1x TBST	100 ml 10x TBS
	900 ml dH_2O
	500 µl Tween
Ponceau S	0.1% Ponceau S (w/v) in 5% (v/v) acetic acid
5% nonfat dried milk	5 g dried non-fat milk powder
	Add up to 100 ml with 1x TBST
High Pure RNA Isolation Kit (Roche)	Lysis/Binding Buffer, DNase I, recombinant, lyophilizate, DNase Incubation Buffer, Wash Buffer I, Wash Buffer II, Elution Buffer
	High Pure Spin Filter Tubes, High Pure Collection Tubes.
First strand cDNA synthesis Omniscript RT Kit (Roche)	Buffer RT, 10x, dNTP Mix, 5 mM each, RNase-Free Water, Omniscript Reverse Transcriptase.

2.1.3 List of devices and materials

Name	Company	Catalogue Number
0.5-20 µl	Eppendorf	6051112
2-20 µl tip	Eppendorf	6051511
2-200 µl tip	Eppendorf	6051441
1250 µl tip	SARSTECT	Z23151
Tissue Culture Plate, 6 well	Life Science	353046
Tissue Culture Plate, 96 well	Life Science	353072
Cell culture flask, 50ml, 25cm2	Greiner Bio-one	690175
Cell Scraper(25cm)	Sarstedt Inc	83.1830
CO_2 incubator	Thermoscientific	BBD 6220
Centrifuge 5415 C	Eppendorf	110110364
Sonifier cell disruptor 250	Brabson Inc	140994
PVDF membranes	Bio-Rad	1620117
Miniplate spinner mps1000	Labnet	K2061299
Powerpac Basic	Bio-Rad	041BR
Semidry transfer Cell	Bio-Rad	221BR12728
Criterion^{TM} Blotter	Bio-Rad	560BR16858

P25 Powerpack	Biometra	D37079
Microcomputer electrophoresis power supply	Consort	3002419
Mini-Protean Tetra System	Bio-Rad	146379
LC480 Multiwell plate	Roth	15030400
Anthos Reader 2001	Anthos	938268
Thermoshaker	Dunn	1104 5464
TPersonal 20 thermocycler	Biometra	100302
Nanophotometer	IMPLEN	100330
LightCycler® 480 II	Roth	5546
Linear accelerator	YXLON	DE228847110

2.1.4 List of antibodies and isotype controls

Name	**Company**	**Catalogue Number**	**Product format**
Rabbit polyclonal anti-Notch1	Santa Cruz Biotechnology	27526	500 µl
Notch3 (8G5) Rat mAb	Cell signaling	3446	100 µl
Rabbit anti-Caspase-3	New England Biolabs	9662s	100 µl
Rabbit anti-Vinculin	Abcam	ab129002	100 µl
Rabbit anti-Tubulin	Abcam	ab185067	100 µl
Rabbit anti-GAPDH	New England Biolabs	3683S	100 µl
Peroxidase-conjugated Affinipure Goat Anit-Rabbit IgG (H+L)	Jackson Immuno Research	116154	2 ml
Peroxidase-conjugated Affinipure Goat Anit-Mouse IgG (H+L)	Jackson Immuno Research	119380	2 ml
Anti-rat IgG, HRP-linked Antibody	Cell signaling	7077	1 ml

2.2 Cell culture

The human HNSCC cell line FaDu were purchased from ATCC (Cat. No: ATCCHTB-43, Manassas, VA, USA). The HNSCC cell lines UD (University of Düsseldorf)-SCC-4, UM (University of Michigan)-SCC-22B were a gift from T.K. Hoffmann (University of Essen) and T.E. Carey (University of Michigan) [94]. Long term exposure (six months) of these cell lines to increasing concentrations of cisplatin resulted in the selection of subclones displaying resistance to cisplatin. FaDu, UD-SCC-4 and UM-SCC-22B, as well as their derived subclones with resistance to cisplatin (FaDu $_{CDDP-R}$, UD-SCC-4 $_{CDDP-R}$ and UM-SCC-22B $_{CDDP-R}$), were cultured in MEM (GIBCO, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum and Non-Essential-Amio-Acid (NEAA, GIBCO, #10270, Grand Island, NY, USA). All cells were cultured in a humidified atmosphere (at 37 °C and 5% CO_2). Cisplatin was purchased from Sigma-Aldrich (Munich, Germany). γ-secretase inhibitor XII (GSI-XII) was purchased from Calbiochem (Darmstadt, Germany), γ-secretase inhibitor IX (GSI-IX, DAPT) from Selleck chemicals (Houston, USA). For in vitro use, both inhibitors were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to a concentration of 10 mmol/L, stored at −80°C, and further diluted to an appropriate final concentration in MEM at the time of use.

2.3 siRNA transfection

Knockdown of Notch1 and Notch3 expression was performed by RNA interference using specific small interfering ribonucleic acid (siRNA) oligonucleotides. The Notch1, Notch3 siRNA and negative control siRNA (non-targeting) were purchased from Dharmacon (GE Healthcare, USA). The sequences of the siRNA used were as follows: Notch1 siRNA (Ref: SO-2470396G): 5'-GCGACAAGGUGUUGACGUU-3', siRNA Notch3 (Ref: SO-2542622G): GUACUGCGCCGACCACUUU-3', negative control siRNA (NTC, Ref: SO-2470400G): UGGUUUACAUGUCGACUAA.

The siRNA transfection was performed with DharmaFECT 1 (Dharmacon, GE Healthcare, USA) according to the manufacturer's protocol. Briefly, tumor cells were seeded in 6-well plates (Fisher Scientific, USA) at a density of 1×10^5 per well. After 24 hours incubation at 37°C, cells were transfected with siRNA against the target gene or with NTC siRNA. The transfection reagent was removed after twenty-four hours. Cells were subjected for gene and protein expression analysis 48 hours after transfection.

2.4 Radiation treatment

HNSCC cells in their exponential growth phase were seeded in a 96-well plate (250 cells/well) and treated with X-ray irradiation at room temperature with a dose rate of 200 cGy/min linear accelerator (YXLON, Hamburg, Germany). Appropriate output dose (2 Gy, 4 Gy or 6 Gy) was delivered under radiation field of 30 cm × 30 cm. Before irradiation, cells were treated with Notch1 siRNA transfection or GSI IX for 24 h. Following irradiation, the plates were then returned to the incubator for a further culture period of 6-8 days. Cells were harvested and subjected for subsequent analysis six to eight days after irradiation.

2.5 Cell viability detection by MTT assay

2.5.1 MTT assay for analysis of the combination of cisplatin and Notch1 siRNA transfection in HNSCC cells

Viability of treated cells and corresponding DMSO controls was measured using MTT assay in six replicates. After being incubated with transfection reagent for 24 h, cells were detached by 0.25% trypsin/EDTA and centrifuged (1500g × 5 min) and then seeded into 96-well plate (250 cells/well). After twenty-four hours, cells were treated with different concentrations of cisplatin. Cells were further incubated in 5% CO_2 incubator for seven days and finally, 20 µl of MTT reagent (5 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide in PBS) was added to each well. After one hour incubation, medium was replaced by DMSO (Roth, Karlsruhe, Germany) and crystal formations were dissolved. Absorption at wavelength of 540 nm was measured using a spectrophotometer. The Proliferation rate of the cells in each group was determined by measuring the optical densities (OD) at 540 nm. Relative viability were calculated based on the control cells. The MTT assay was performed at least three independent experiments. SPSS 22.0 software was applied to calculate half inhibitory concentration of each group (IC50). Drug resistance index (DRI) was calculated = IC50 of Notch1 siRNA treated cells/IC50 of NTC siRNA treated cells [95].

2.5.2 MTT assay for analysis of the combination of radiation and Notch1 siRNA transfection in HNSCC cells

After being incubated with transfection reagent for 24 h in 6-well plate, cells were detached by 0.25% trypsin/EDTA and centrifuged (1500g × 5 min) and then seeded into 96-well plate (250 cells/well). Twenty-four hours after transfection, cells were irradiated with 0, 2, 4, and 6 Gy of X-rays at room temperature. Cells were cultured for a further six to eight days before MTT proliferation assay. The MTT assay was performed in at least three independent experiments. IC50 was also calculated. Viability of treated cells and non-treated cells were measured in six replicates.

2.5.3 MTT assay for analysis of the combination of cisplatin and GSI IX treatment in HNSCC cells

Cancer cells (250 cells/well) were seeded in a 96-well flat-bottom plate. After twenty-four hours, cells were treated with different concentration of GSI IX. Cisplatin was added 24 h after GSI IX treatment. Cells were further incubated in 5% CO_2 incubator for 7days. At the end, 20 µl of MTT reagent (5 mg/ml) was added to each well. After one hour incubation, medium was replaced by DMSO (Roth, Karlsruhe, Germany) and crystal formations were dissolved. Absorption at wavelength of 540 nm was measured using a spectrophotometer. Relative viability were calculated based on the DMSO control cells. The MTT assay was performed in at least three independent experiments. IC50 was also calculated. DRI was calculated = IC50 of GSI IX treated cells/IC50 of DMSO-treated cells [95].

2.5.4 MTT assay for analysis of the combination of radiation and GSI IX treatment in HNSCC cells

HNSCC cells during exponential growth phase were seeded in 96-well plate (250 cells/well). Twenty-four hours after seeding, cells were treated with 30 µM GSI IX or same volume of DMSO. One day after GSI IX treatment, cells were irradiated with 0, 2, 4, and 6 Gy of X-rays. The plates were then returned to the incubator for further culture (Six to eight days). At the end, 20 µl of MTT reagent (5 mg/ml) was added to each well. After one hour incubation, medium was replaced by DMSO (Roth, Karlsruhe, Germany) and crystal formations were dissolved. Absorption at wavelength of 540 nm was measured using a spectrophotometer. Relative viability were calculated based on the DMSO control cells. The MTT assay was performed in at least three independent experiments. IC50 was calculated.

2.6 Western blot

For harvesting the cells to be analyzed medium was removed from the plate and the cell monolayer was washed with Dulbecco's Phosphate Buffered Saline (PBS, GIBCO, Grand Island, NY, USA) two times. Cell pellets were harvested on ice using RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo scientific, Waltham, MA, USA) as well as 2 mM EDTA (Thermo scientific, Waltham, MA, USA). Ultrasonic processing of this solution was then carried out using an ultrasonic processor (Sonifier cell disruptor 250, Brabson Inc, USA). The lysates were collected, centrifuged at 12 000 revolutions per minute (rpm) for 20 minutes at 4°C, and the supernatant was collected. The total protein concentration was quantified by the BCA (bicinchoninic acid) protein assay kit (Thermo scientific, Waltham, MA, USA).

For western blotting, equal amounts of proteins (25-80 µg) were separated using 6-12% SDS-polyacrylamide gel. After gel electrophoresis, proteins were transferred onto Immuno-Blot PVDF membranes (Bio-Rad, CA, USA) for immunostaining. After blocking with 5% non-fat milk (Bio-Rad, CA, USA) in 1 × TBST (10 mM Tris, 150 mM NaCl, PH 8.0, and 0.1% Tween) for one hour at room temperature, membranes were incubated in primary antibody overnight at 4°C. Anti-Notch1 (1:750, Santa Cruz, CA, USA) was used to visualize full-length Notch1 (330 kDa) and cleaved Notch1 (130 kDa). Anti-Notch3 (1:750, Cell signaling, MA, USA) was used to visualize full-length Notch3 (270 kDa) and cleaved Notch3 (95 kDa). A caspase-3 antibody (1:750, Cell signaling, MA, USA) was used to recognize the active caspase-3 fragment (17-20 kDa) as well as full-length caspase-3 (37 kDa). Antibody Vinculin (1:1000, Abcam), alpha-tubulin (1:1000, Abcam) and GAPDH (1:1000, New England Biolabs) were used as a loading control to detect the protein levels of Vinculin, alpha-tubulin and GAPDH, respectively. After being washed in 1 × TBST three times (10 minutes per time), the membrane was incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell signaling, MA, USA) diluted 1:15 000 in 5% non-fat milk for 1 h at room temperature and then washed three times in TBST. For detecting Notch3, anti-rat HRP-linked secondary antibody (Cell signaling, MA, USA) diluted 1:1 000 in 5% non-fat milk was applied. The blots were developed using ECL plus Western Blotting Detection Reagents (Thermo scientific, Waltham, MA, USA). The protein signals were exposed to FUJI medical X-ray film (FUJIFILM, FUJI, JAPAN). Vinculin, alpha-tubulin and GAPDH was used to normalize the protein expression. Intensity of bands for different proteins was quantified with Image J 1.50 software after EPSON stylus TX130 scanning.

2.7 RNA isolation and cDNA synthesis

Total RNA was extracted from cells using the High Pure RNA Isolation Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Nanophotometer (IMPLEN, Munich, Gernamy) was used to measure the RNA concentration. RNA quality and purity was assessed by the OD260/OD280 nm absorption ratio, which supposed to in the range between 1.9 and 2.1. Five hundred nanogram of total RNA was used for complementary DNA (cDNA) synthesis using first strand cDNA synthesis Omniscript RT Kit (Qiagen, Silicon Valley, CA, USA) for RT-PCR according to the manufacturer's protocol. 25 µl of cDNA was prepared of each sample by TPersonal 20 thermocycler (Biometra, Göttingen, Germany) using 1 cycle of 60 min at 37°C.

2.8 Real-time RT-PCR

Real-time RT-PCR was performed by using LightCycler® instrument (Roche Diagnostics, Germany) and results were analyzed by LC software release 3.0. The crossing point (Cp) values without baseline adjustments were recorded in all samples. Amplification was done in 20 µl volume, including 2.0 µl first strand cDNA, 4 nM of each primers, 2 nM of universal probe and 2x LightCycler 480 DNA master hybridization mix. Real-time RT-PCR primer pairs and their appropriate probe were designed using the Assay Design Center Web Service (http://qpcr.probefinder.com/roche3.html). The cycling variables were one cycle of 10 minutes at 95°C initial denaturing, 50 cycles of 10 seconds at 95°C, 30 seconds at 60°C and 1 second at 72 °C for amplification, and final cooling step at 40°C. The primer and probe sequences used in this study have been shown in Table 1. The TUBA1C mRNA, a widely accepted standard, was quantified to adjust the amount of mRNA in each sample. Relative gene expression to *TUBA1C* was calculated as $2^{-\Delta\Delta Cp}$, with ΔCp determined by subtracting the average housekeeping gene TUBA1C threshold cycle from the average target gene value.

Table 2.1 Primer sequences and UPL probes used for the real-time RT-PCR

Target gene	Amplicon size, nt	Forward primer, 5'-3'	Reverse primer, 5'-3'	UPL probe
NOTCH1	96	CAGCCAGTGCAACTCAAGC	TCCTTGCAGTACTGGTCGTACA	34
NOTCH2	91	AAGGAACCTGCTTTGATGACA	CAGGGAGCCAATACTGTCTGA	59
NOTCH3	105	TGGCATGGATGTCAATGTG	GCCTCATCCTCTTCAGTTGG	50
NOTCH4	118	AGGCTGCACTGAGCCAAG	CACAGGCTGCCTTGGAAT	59
HEY1	83	CATACGGCAGGAGGGAAAG	GCATCTAGTCCTTCAATGATGCT	29
TUBA1C	74	CCCCTTCAAGTTCTAGTCTGC	GCATTGCCAATCTGGACAC	58

Note: UPL: Universal probe library.

2.9 Statistics

Statistical analysis was performed with SPSS v.22.0 (IBM Corp, Armonk, NY, USA) software. Data are expressed as mean ± standard deviation (SD). Expression levels of the two groups (Such as the expression of Notch1 mRNA in FaDu cells vs FaDu $_{CDDP-R}$ cells) were compared by using the independent samples t‾test. Values of several groups were compared using Student-Newman-Keuls' (SNK) test in post-hoc testing of one-way ANOVA. A $p<0,05$ was considered to indicate a statistically significant difference.

3. Results

3.1 *NOTCH1* gene mutations in HNSCC cell lines

In a previous project in our lab, the mutation status of the six HNSCC cell lines was examined by a targeted Next-Generation-Sequencing (NGS) approach. The alteration of *NOTCH1* mutations between the parental cell lines and the derived cisplatin resistant subclones was investigated. As shown in Table 3.1, *NOTCH1* gene mutations in UM-SCC-22B and UM-SCC-22B $_{CDDP-R}$ were nonsense mutations in the *NOTCH1* coding gene (p.E1679X) that results in premature translation termination of Notch1 protein, which was consistent with the result of western blot (Figure 3.1A). No mutations were identified in FaDu, UD-SCC-4 and their resistant subclones. For all three cell line pairs no changes in *NOTCH1* gene status between parental cell lines and their derived cisplatin resistant subclones were observed.

Table 3.1 *NOTCH1* gene mutations identified by panel Next-Generation Sequencing for HNSCC cell lines

Cell line	Notch1 protein mutation	Effect of mutation
FaDu	WT	-
FaDu $_{CDDP-R}$	WT	-
UD-SCC4	WT	-
UD-SCC4 $_{CDDP-R}$	WT	-
UM-SCC-22B	Glu1679Ter	Nonsense
UM-SCC-22B $_{CDDP-R}$	Glu1679Ter	Nonsense

Note: WT, wide type

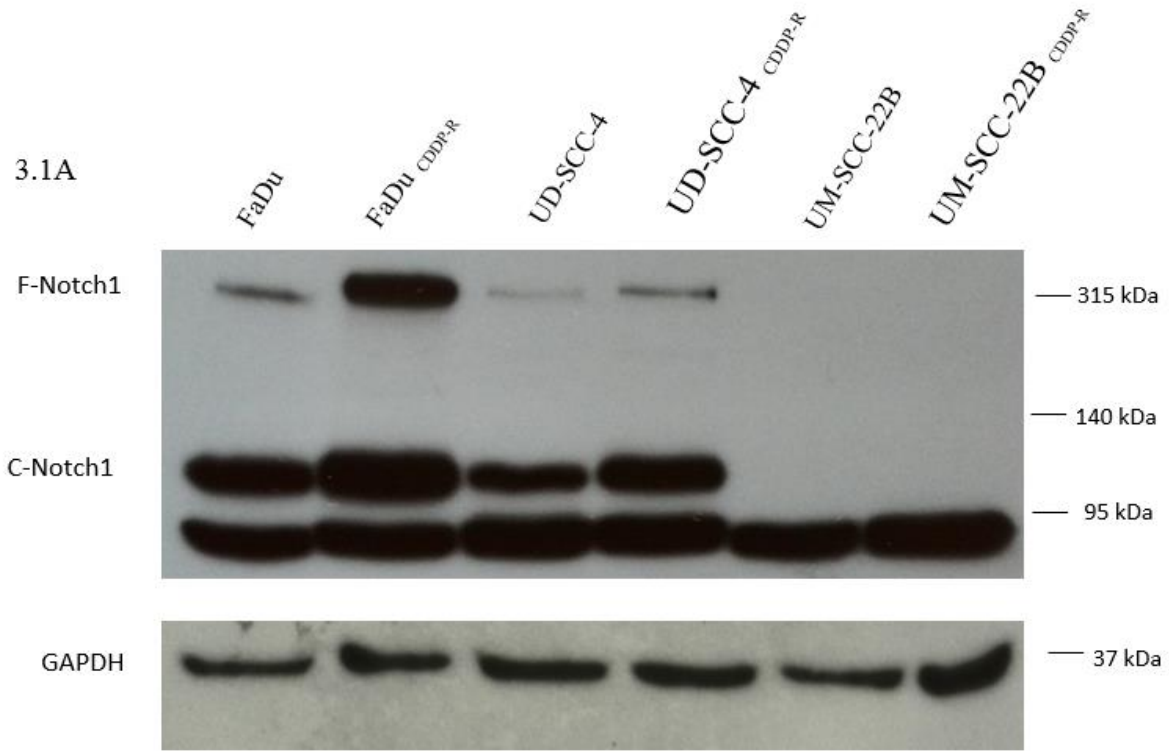
3.2 Basal Notch1 expression in HNSCC cell lines

The objective of this part of the project was to determine whether Notch1 might be involved in cisplatin resistance. First, the basal levels of Notch1 expression in these three pairs of HNSCC lines were evaluated at protein and mRNA levels. To compare the protein expression levels between the sensitive and resistant cell lines immunoblotting was performed. Therefore, cells growing in a 10-cm cell culture dish to a density of 80 % were harvested. Ultrasonic processing was then carried out and protein lysates were collected. The lysates were centrifuged and the supernatant was collected. Protein lysates (80 µg per sample) were subjected to SDS-PAGE and western blot analysis. For comparison of different HNSCC cell lines sensitive and resistant cells

of FaDu, UD-SCC-4 and UM-SCC-22B were examined for Notch1 protein expression. The experiments were performed in three independent experiments.

The results of western blot (Figure 3.1A and 3.1B) indicated that FaDu $_{CDDP-R}$ expressed the highest level of full-length (F-Notch1) and cleaved Notch1 (C-Notch1, as well as active Notch1), while there was no Notch1 protein expressed in UM-SCC-22B and UM-SCC-22B $_{CDDP-R.}$ This could be due to the identified nonsense mutation in the *NOTCH1* coding gene (p.E1679X) which might lead to degradation of the truncated Notch1 protein. FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ expressed higher levels of F-Notch1 and C-Notch1 compared to their isogenic sensitive parental cells (FaDu and UD-SCC-4).

There was an additional band with a molecular weight of 90 kDa, which could be observed in each sample. Since the UM-SCC-22B cell lines showed the same protein band though lacking F-Notch1 and C-Notch1, this band was considered unspecific.



3.1B

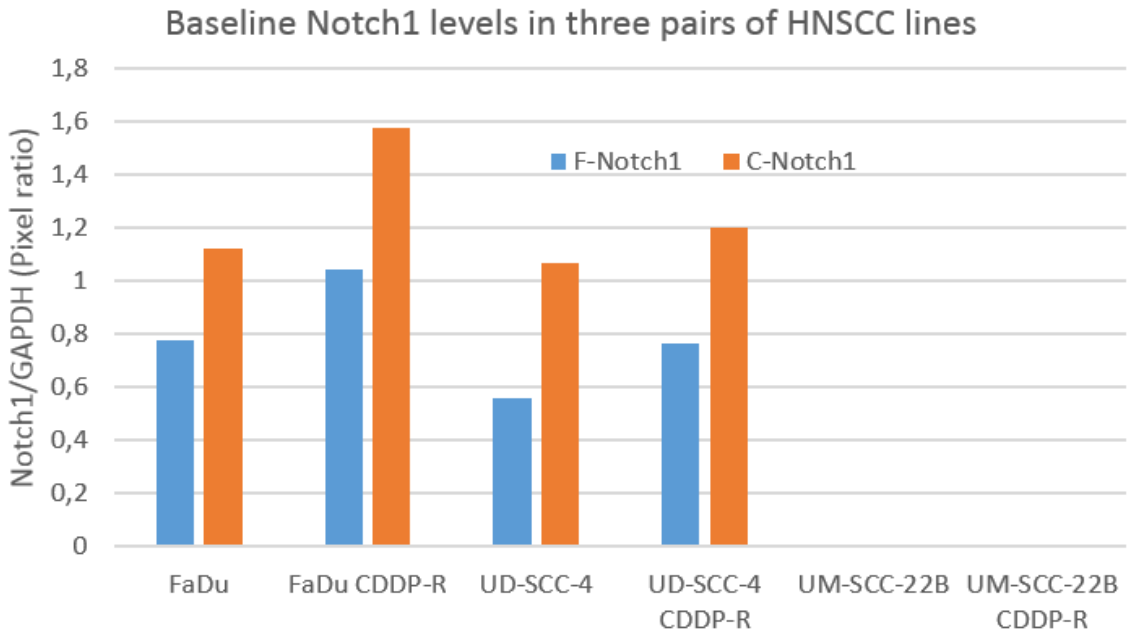


Figure 3.1: Notch1 overexpression might participate in cisplatin resistance in HNSCC cells. A) The basal level of Notch1 expressions in the three pairs of HNSCC lines. B) The band intensity of Notch1 was quantitated by Image J 1.50 software and the ratio of each band relative to GAPDH signal was determined. The relative Notch1 protein expression levels are presented.

Furthermore, the mRNA expression levels of Notch1 were evaluated in these three pairs of HNSCC cell lines. Again, cells cultured in a 10-cm dish to 80 % confluence were harvested and subjected to isolation of total RNA. After cDNA synthesis, quantitative real time RT-PCR was performed. The relative expression of Notch1 normalized firstly to the endogenous control TUBA1C (Tubulin) and secondly to the expression levels of FaDu was calculated. As shown in Figure 3.2, Notch1 mRNA expression in FaDu was the highest among the six cell lines. The Notch1 expression in UD-SCC-4 $_{CDDP-R}$ was twice as high as in UD-SCC-4 (p=0.040). Similarly, there was more mRNA levels of Notch1 in UM-SCC-22B $_{CDDP-R,}$ compared with UM-SCC-22B, but this difference did not reach statistical significance (p=0.186). The Notch1 mRNA expression was almost comparable between FaDu and FaDu $_{CDDP-R}$ (p=0.103). These results can be explained by different mechanisms of Notch1 upregulation in FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ cells.

Since Notch1 protein was overexpressed in cisplatin-resistant compared to cisplatin-sensitive cells in the FaDu and UD-SCC-4 cell lines, these two models were selected for further functional evaluation of the role of Notch1 in cisplatin sensitivity.

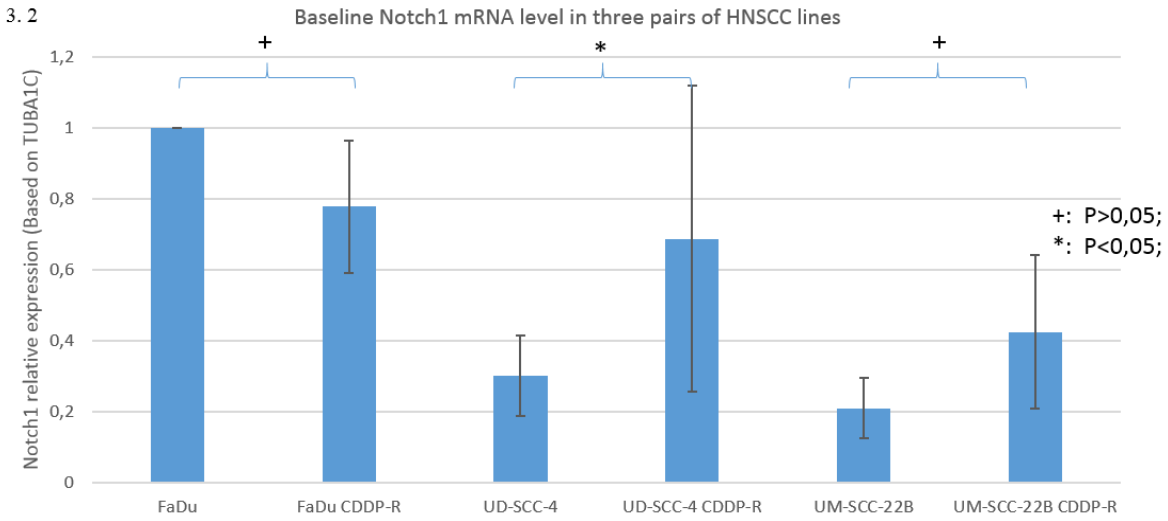


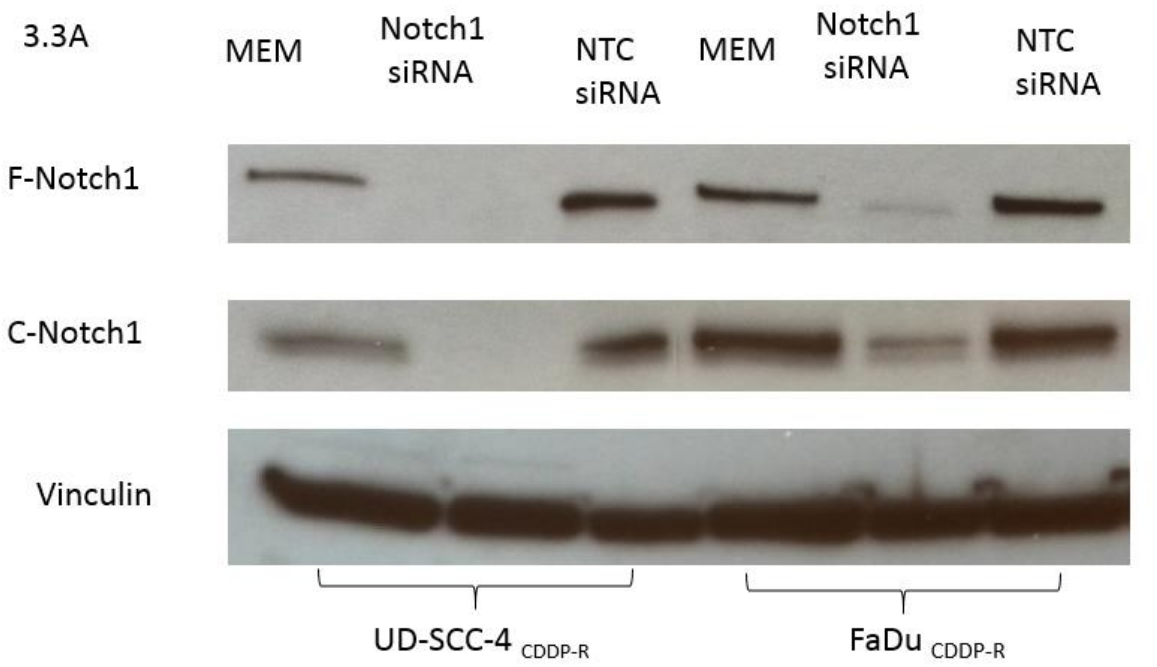
Figure 3.2: The basal levels of Notch1 mRNA expression: The gene expression normalized to TUBA1C and relative to the Notch1 mRNA level in FaDu is shown.

3.3 Knock down of Notch1 by siRNA in HNSCC cell lines

FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ showed highest protein expression of Notch1 in the four examined cell lines (Figure 3.1A). Therefore, we choose FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ for the establishment of Notch1 knock down by siRNA. First I needed to find out which concentration and time point of transfection could be selected for subsequent experiments.

FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ were transfected with different concentrations of Notch1 siRNA or NTC siRNA in a 6-well plate and incubated at 37°C for 48-96 hours. Cells were harvested and subjected to western blot analysis at different time points (data not shown). In the first experiments, an optimal concentration of 25 nM siRNA and an incubation time of 48 hours were established. These experimental conditions were then for further siRNA knock down experiments. As shown in Figure 3.3A and 3.3B, FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ cells transfected with Notch1 siRNA displayed decreased expression levels of F-Notch1 and C-Notch1 proteins. In contrast, NTC siRNA did not affect Notch1 expression. The western blot results indicated that the transfection specifically reduced the protein expression of F-Notch1 as well as its activated cleavage product (C-Notch1). We observed a 95% decrease in Notch1 protein expression in FaDu $_{CDDP-R}$, whereas a 85% decrease in F-Notch1 and 67% decrease in C-Notch1 was observed in UD-SCC-4 $_{CDDP-R}$. Furthermore, the suppression of Notch1 by siRNA was confirmed by real-time RT-PCR analysis. As housekeeping gene *TUBA1C* was used. The results showed that the efficiency of Notch1 silencing was >75% in FaDu and UD-SCC-4 and about 50%

in their cisplatin-resistant subclones at 48 hours post-transfection (Figure 3.4A). Compared to NTC siRNA, Notch1 mRNA levels were markedly reduced at 96 hours after Notch1 siRNA transfection (Figure 3.4B).



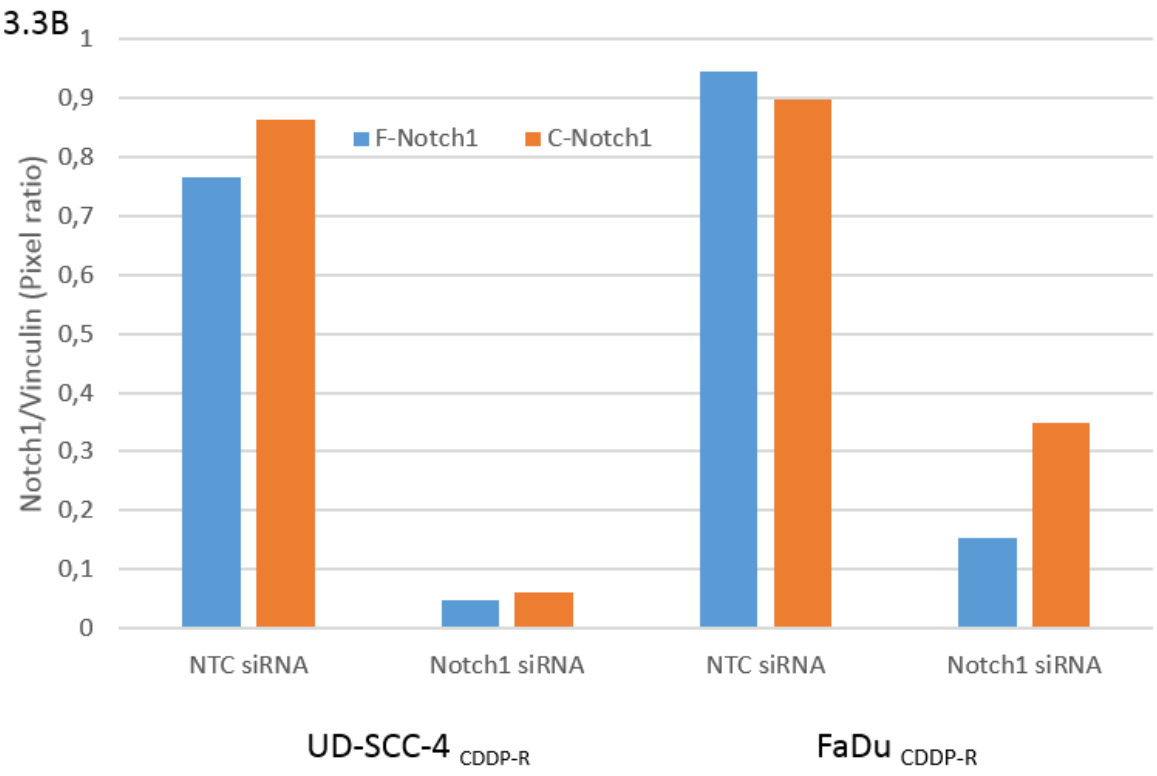


Figure 3.3: Downregulation of Notch1 protein expressions by Notch1 siRNA (25 nM). A) Forty-eight hours post-transfection, Notch1 protein expressions of control (MEM), Notch1 siRNA transfected and NTC siRNA transfected

cells were evaluated by western blot. B) The band intensity of Notch1 was quantitated, normalized to vinculin expression and expressed as relative Notch1 protein expression levels.

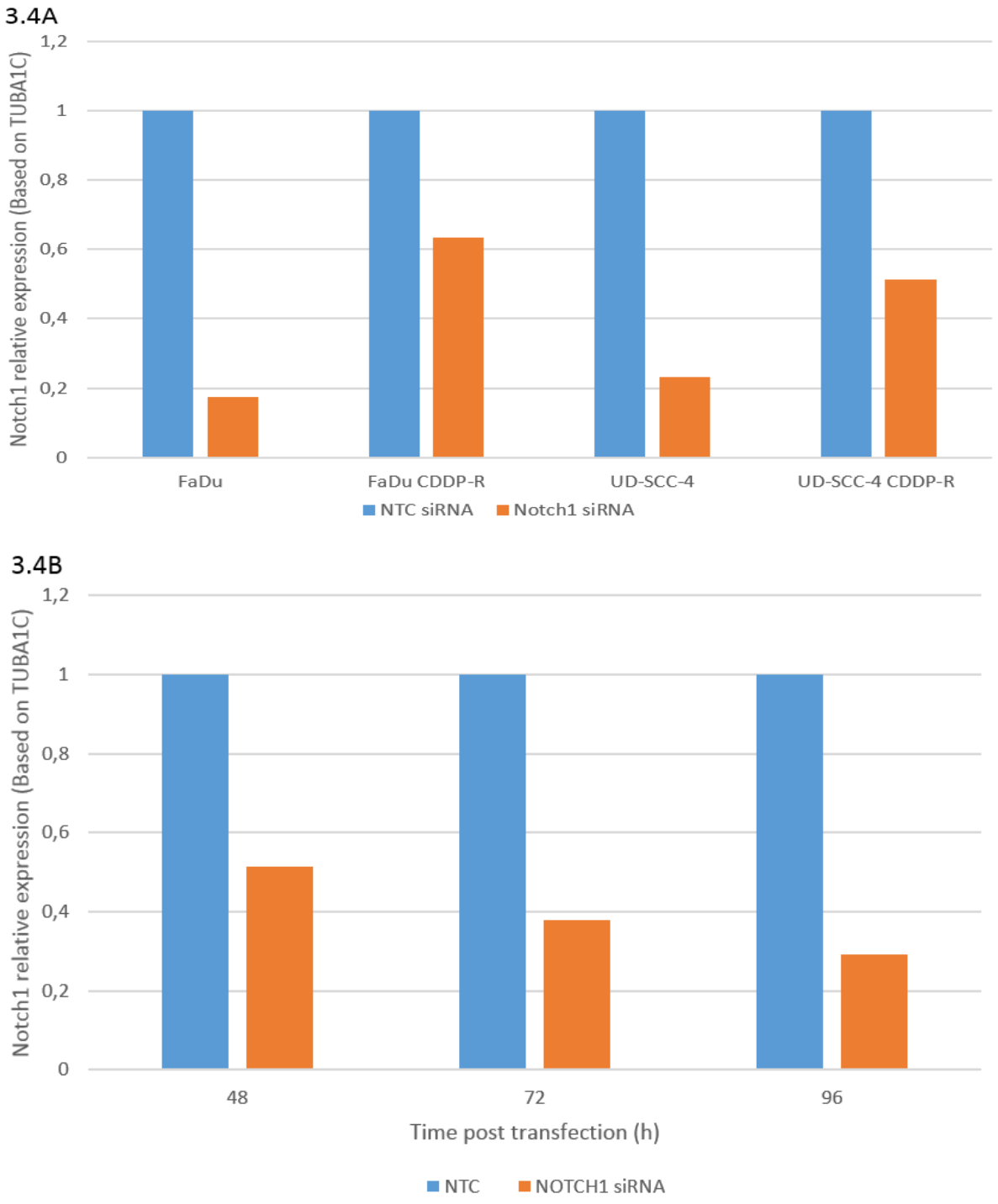


Figure 3.4: A) The effect of Notch1 siRNA was confirmed by real-time RT-PCR in FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ cells and their cisplatin-sensitive parental cells. B) Compared to NTC siRNA, the Notch1 siRNA transfection markedly reduced the expression of Notch1 at mRNA level in a time-dependent manner.

3.4 Effect of Notch1 siRNA transfection on the expression of its target genes

HES1 and *HEY1* are two of the most common target genes of the Notch signaling pathway. Thus, the next aim was to evaluate if Hes1 and Hey1 expression could be decreased as well after Notch1 siRNA transfection. Real-time RT-PCR was performed in UD-SCC-4 cells, in which Notch1 expression was successfully knocked down by Notch1 siRNA. UD-SCC-4 cells were harvested at 48h, 72h and 96h after siRNA transfection. Total RNA was extracted for real time RT-PCR. Compared to the NTC siRNA group, Notch1-knockdown in UD-SCC-4 cells did not significantly reduce expression of Hes1 and Hey1 (Shown in Figure 3.5). In contrast, 72h or 96h after transfection the expression of Hes1 was even slightly increased.

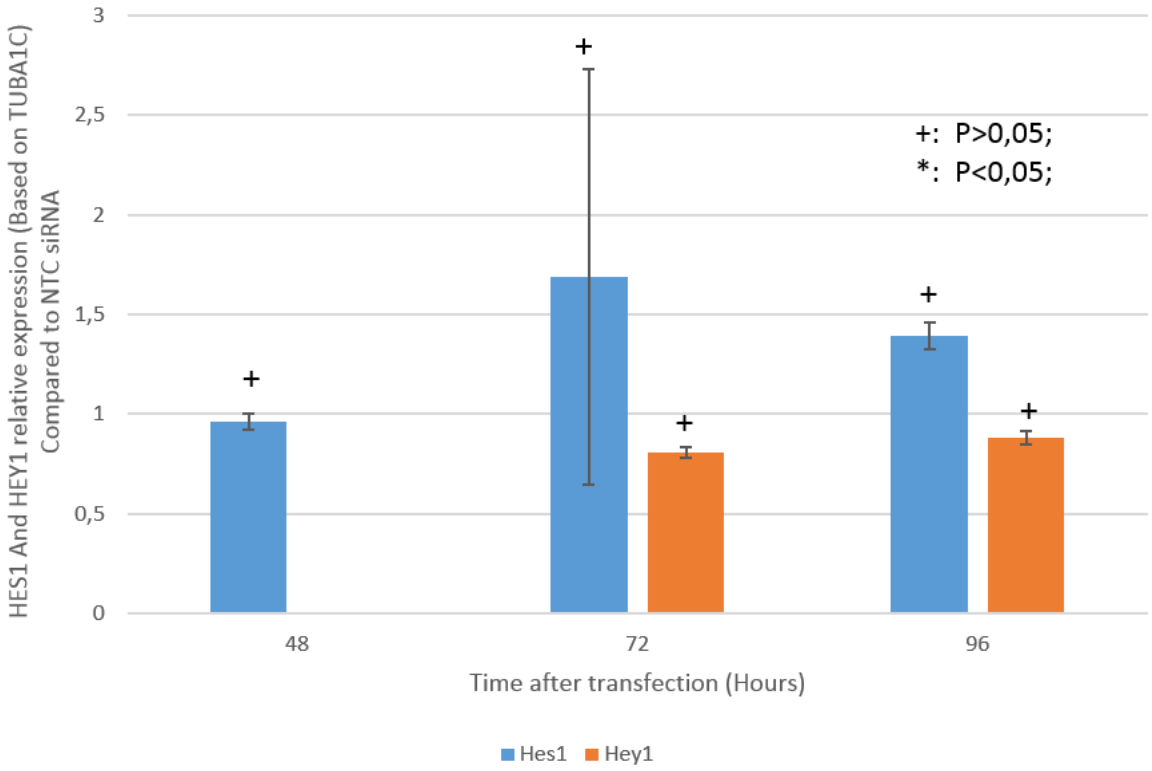


Figure 3.5: Relative gene expression of the Notch1 target genes Hes1 and Hey1, normalized to TUBA1C and relative to the value in NTC siRNA group. This figure demonstrated the changes of Hes1 and Hey1 expression at 48h, 72h and 96h after transfection, determined by real-time RT-PCR. Relative gene expression of Hey1 was not measured at 48 hours after transfection. Results from three independent experiments are shown.

3.5 Effect of Notch1 knockdown by siRNA on cisplatin sensitivity

Since Notch1 siRNA transfection effectively reduced expression of Notch1, we intended to determine whether this would affect the cisplatin sensitivity of HNSCC cell lines. In this project

part the sensitivity of Notch1 siRNA treated cells to long-term treatment with cisplatin was compared to NTC siRNA treated cells using the MTT assay. For this approach, treatment with cisplatin took place six to eight days. The concentrations of cisplatin used for FaDu and FaDu $_{CDDP-R}$ ranged from 0 to 0.16 µg/ml and 0 to 0.40 µg/ml, respectively. The concentrations used for UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ were 0 to 0.40 µg/ml and 0 to 2.4 µg/ml, respectively. As shown in Figure 3.6, dose-response curves from MTT assays demonstrated no difference in the relative viability of cells from the Notch1 siRNA treated group and the control group in these four cell lines ($p>0.05$). Furthermore, the IC50 values and DRI index were similar the two groups in these four cell lines (Table 3.2, $p>0.05$). In summary, the results provided evidence that inhibition of Notch1 by siRNA knockdown did not change sensitivity to cisplatin in these four HNSCC cell lines. Furthermore, the finding of higher IC50 and DRI in FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ cells in contrast to the sensitive parental cells ($p<0.05$, Table 3.2) confirmed the cisplatin resistance of FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$.

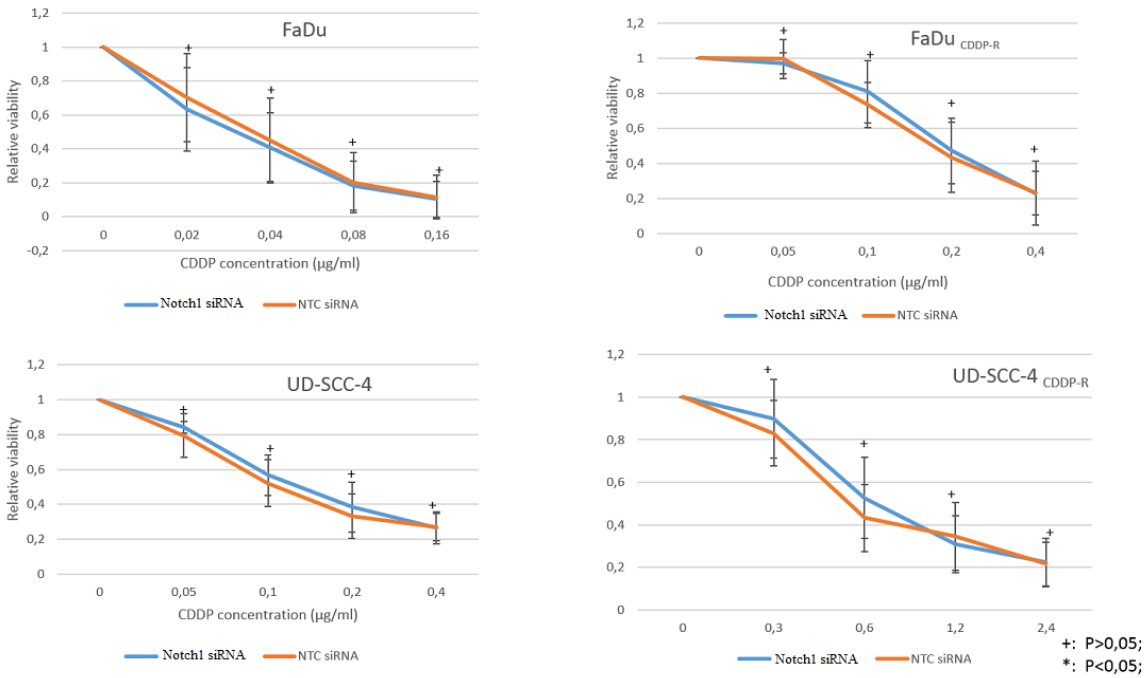


Figure 3.6: Effects of Notch1 siRNA transfection on cisplatin sensitivity in four HNSCC cell lines as assessed by MTT assay. The relative viability of the cells in each group was determined by measuring the optical densities (OD) at 540 nm. The results from three independent experiments are shown.

Table 3.2 Determination of IC50 and DRI of cisplatin in HNSCC cells treated with Notch1 siRNA and NTC siRNA (mean ± SD)

Cell line	IC50 of cisplatin (μg/ml)		DRI	p value
	Notch1 siRNA	NTC siRNA		
FaDu	0.032±0.017	0.040±0.031	0.800	0.639
FaDu $_{CDDP-R}$	0.215±0.077	0.207±0.139	1.039	0.913
UD-SCC-4	0.161±0.078	0.137±0.062	1.175	0.672
UD-SCC-4 $_{CDDP-R}$	0.828±0.302	0.784±0.355	1.056	0.887

Note: IC50= Half inhibitory concentration; DRI= Drug resistance index.

3.6 Effect of Notch1 knockdown by siRNA on radiation sensitivity

Next, we intended to determine whether Notch1 knockdown by siRNA can affect the radiation sensitivity of HNSCC cell lines. After being incubated with transfection reagent for 24 h in 6-well plate, cells were detached by 0.25% trypsin/EDTA, centrifuged (1500g × 5 min) and seeded into 96-well plate (250 cells/well). Doses of 0 to 6 Gy were applied to all cells twenty-four hours after Notch1 siRNA transfection. Subsequently, long-term MTT assay with six to eight days culture after irradiation was performed. As shown in Figure 3.7, dose-response curves from MTT assays demonstrated no difference in survival rates between non-treafected cells (MEM group), cells transfected with Notch1 siRNA or NTC siRNA in these four cell lines ($p>0.05$). Furthermore, the IC50 values and DRI index of the irradiation were similar between the Notch1 siRNA group and the NTC siRNA group in these four cell lines (Shown in Table 3.3, $p>0.05$). Furthermore, there was no significant difference for IC50 values for radiation between the sensitive FaDu and UD-SCC-4 cell lines and their resistant derivatives (all $p>0.05$). Thus, long-term treatment of FaDu and UD-SCC-4 with cisplatin only resulted in cisplatin resistance, but did not induce cross-resistance to radiation.

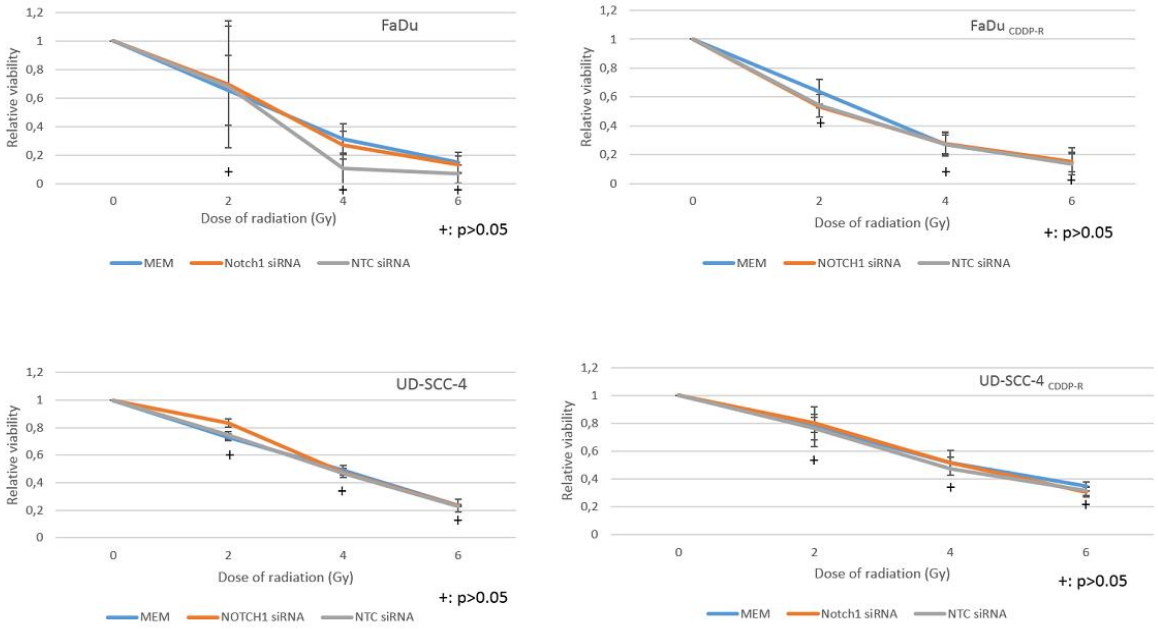


Figure 3.7: Effects of Notch1 siRNA transfection on radiation sensitivity in four HNSCC cell lines. The relative viability of the cells in each group was determined by long-term MTT assay. Results from three independent experiments are shown.

Table 3.3 Determination of IC50 and RI (resistance index) of radiation in HNSCC cells treated with Notch1 siRNA and NTC siRNA (mean ± SD)

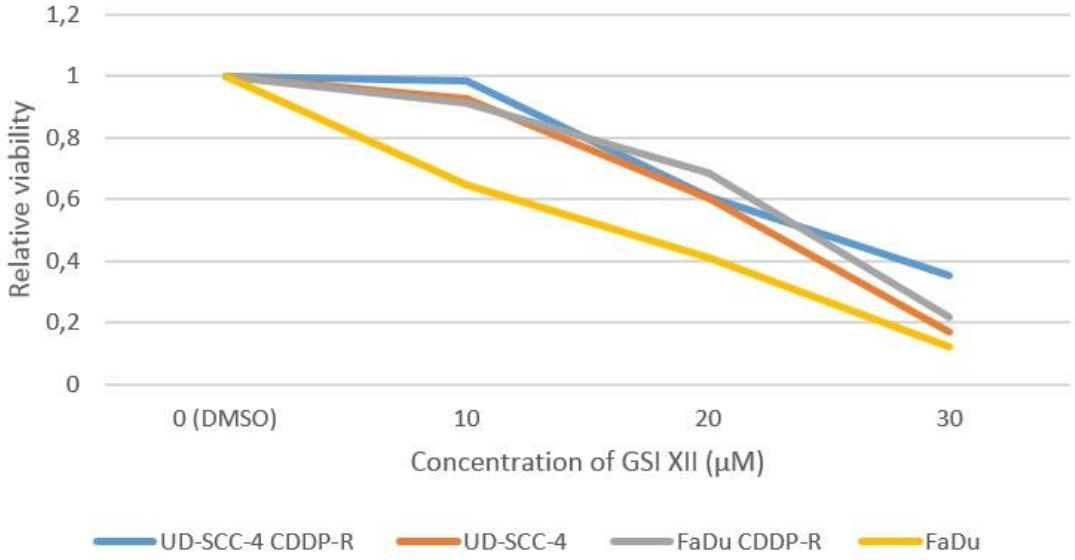
Cell line	IC50 of radiation (Gy)			RI	P value
	MEM	Notch1 siRNA	NTC siRNA		
FaDu	2.71±1.02	2.29±0.90	2.31±1.01	0.991	0.981
FaDu $_{CDDP-R}$	2.54±0.33	2.19±0.05	2.13±0.40	1.028	0.799
UD-SCC-4	3.55±0.30	3.79±0.14	3.51±0.12	1.080	0.256
UD-SCC-4 $_{CDDP-R}$	4.08±0.86	4.06±0.22	3.81±0.47	1.065	0.463

Note: Resistance index.

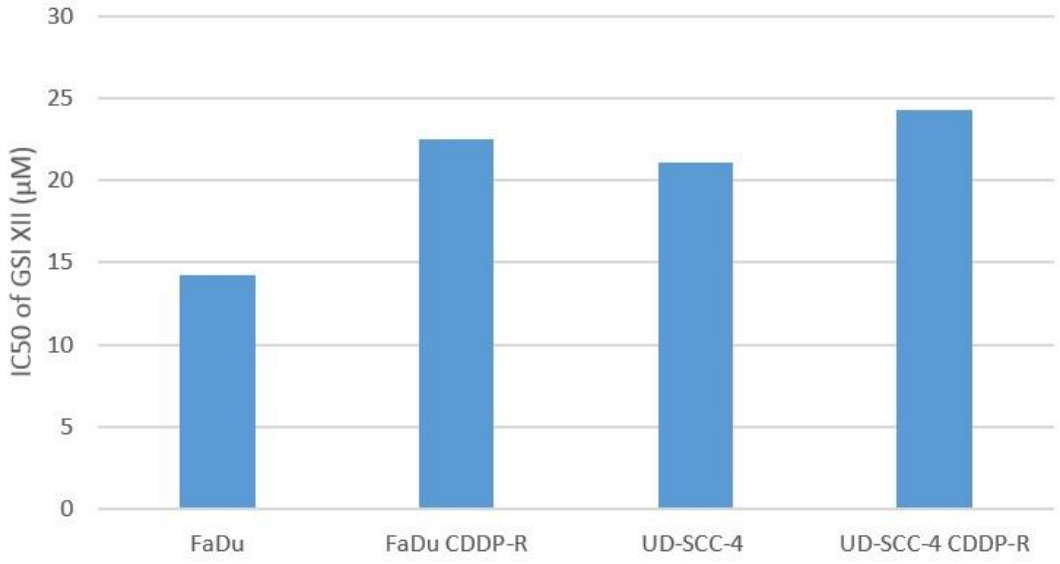
3.7 The effect of GSI XII and GSI IX on cell proliferation and cleaved Notch1 in HNSCC cell lines

Since siRNA-mediated knock down of Notch1 did not change cisplatin sensitivity in HNSCC cells, I decided to investigate whether the inhibition of the Notch1 signaling pathway by GSI could alter cisplatin sensitivity. Therefore, the effects of GSI itself on HNSCC cell proliferation and Notch1 activation by cleavage was evaluated firstly. Long-term MTT assay was used for this approach to check the influence on cell proliferation. Briefly, 250 tumor cells were seeded in 96-well culture plates, allowed to adhere overnight, and treated with increasing concentrations of GSI or DMSO as solvent control (the same volume as used for a final GSI concentration of 30 µM). After seven days, MTT assays were carried out. Dose-response curves showing the effects of GSI XII on the growth of four HNSCC cells lines are presented in Figure 3.8A. The growth of FaDu, FaDu $_{CDDP-R}$, UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ cells were suppressed by GSI XII in a dose-dependent manner. The IC50 for FaDu, FaDu $_{CDDP-R}$, UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ were 14.27 µM, 22.53 µM, 21.10 µM and 24.24 µM, respectively (Figure 3.8B). However, the proliferation of FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ was not affected by GSI IX (Figure 3.8C). Therefore, we further examined the cisplatin sensitizing effects of GSIs, focusing on GSI IX in these four cell lines.

3.8A


1,2
1
0,8
0,6
0,4
0,2
0
Relative viability
0 (DMSO)
10
20
30
Concentration of GSI XII (μM)
UD-SCC-4 CDDP-R
UD-SCC-4
FaDu CDDP-R
FaDu

3.8B


30
25
20
15
10
5
0
IC50 of GSI XII (μM)
FaDu
FaDu CDDP-R
UD-SCC-4
UD-SCC-4 CDDP-R

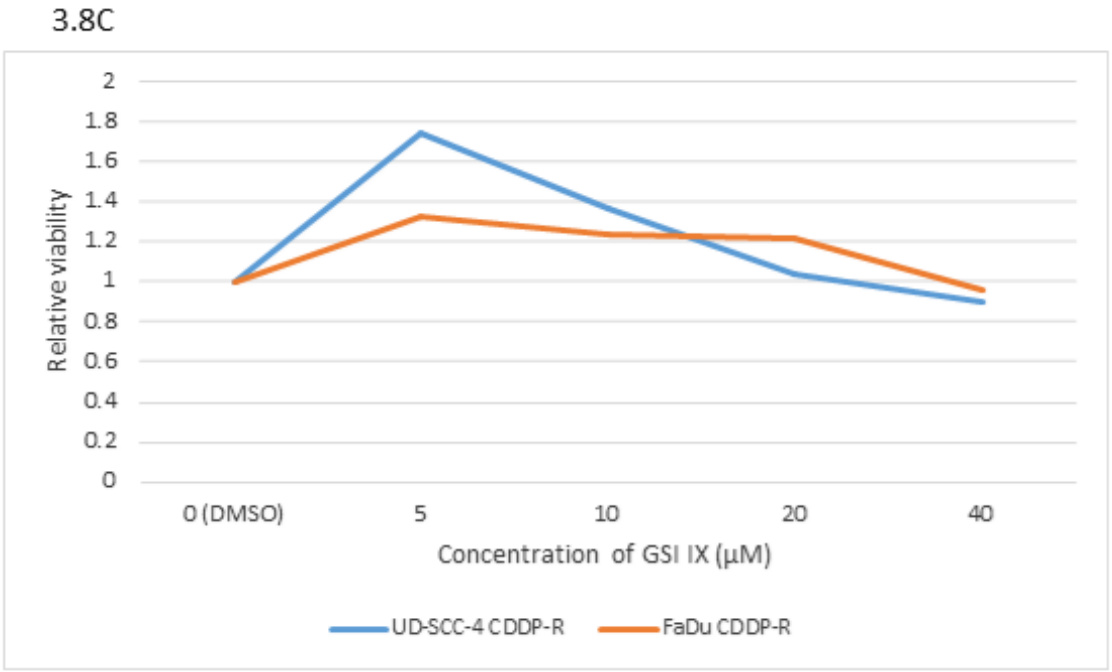


Figure 3.8: Dose-response curves for GSI XII (A) and GSI IX (C) in HNSCC cells. B) The IC50 for GSI XII in FaDu, FaDu $_{CDDP-R}$, UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ were shown.

Then, the effect of GSIs on expression of the activated form of Notch1 (NICD1, Notch1 intracellular domain 1) was assessed by western blot. Cells were treated with different doses of the two GSIs in 6-well plates. 72 hours after treatment, cells were harvested and proteins lysates were generated for western blot analysis. First, C-Notch1 was measured in FaDu $_{CDDP-R}$. As shown in Figure 3.9A and 3.9B, GSI XII treatment did not affect the C-Notch1 expression. Compared with the control group, protein levels of C-Notch1 in FaDu $_{CDDP-R}$ were decreased in dose-dependent manner by GSI IX. Treatment with GSI IX at 30 µM resulted in more than 50% inhibition of C-Notch1 expression. Based on the results of MTT assay and western blot, all further experiments were performed with GSI IX at 30 µM.

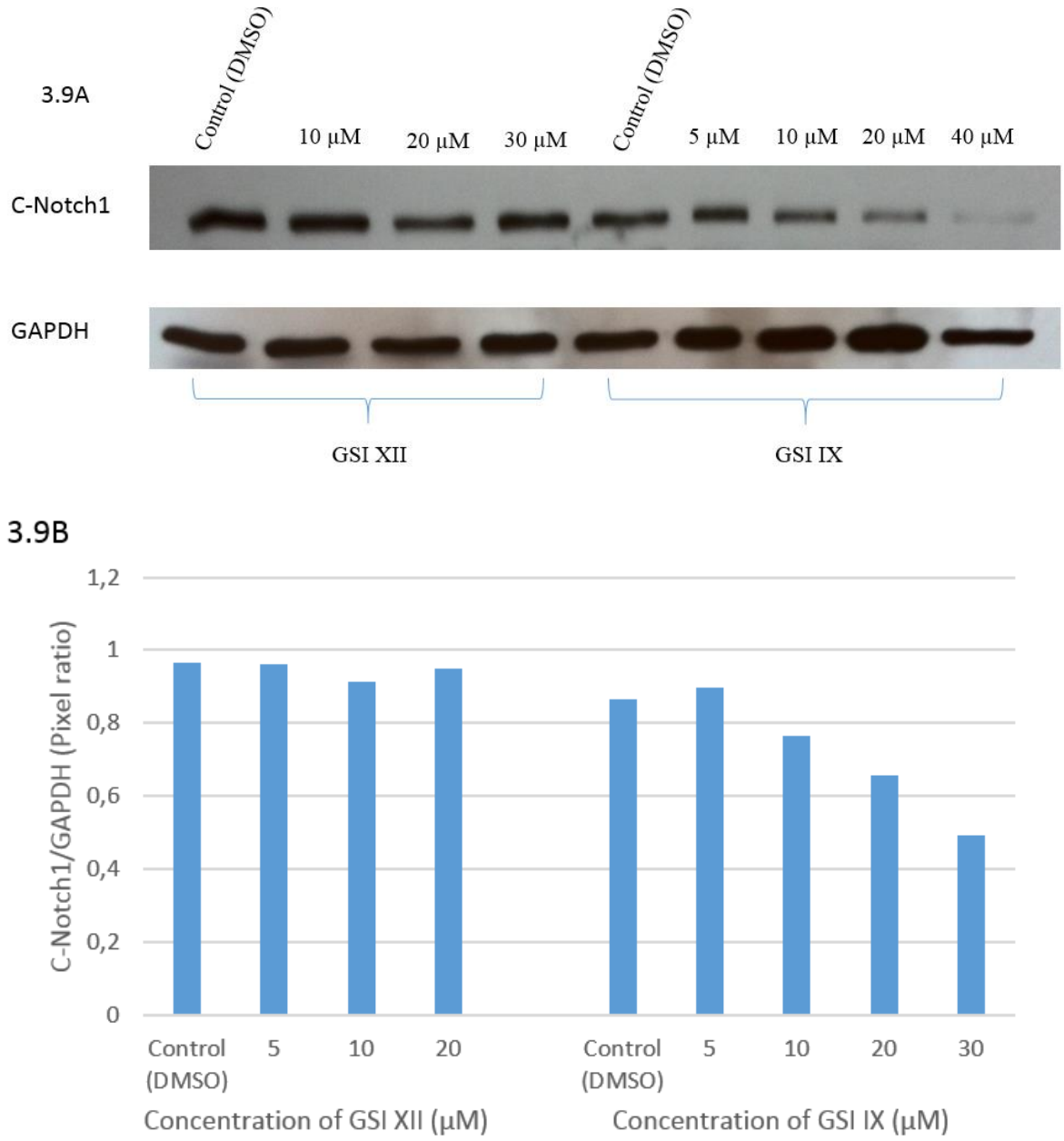
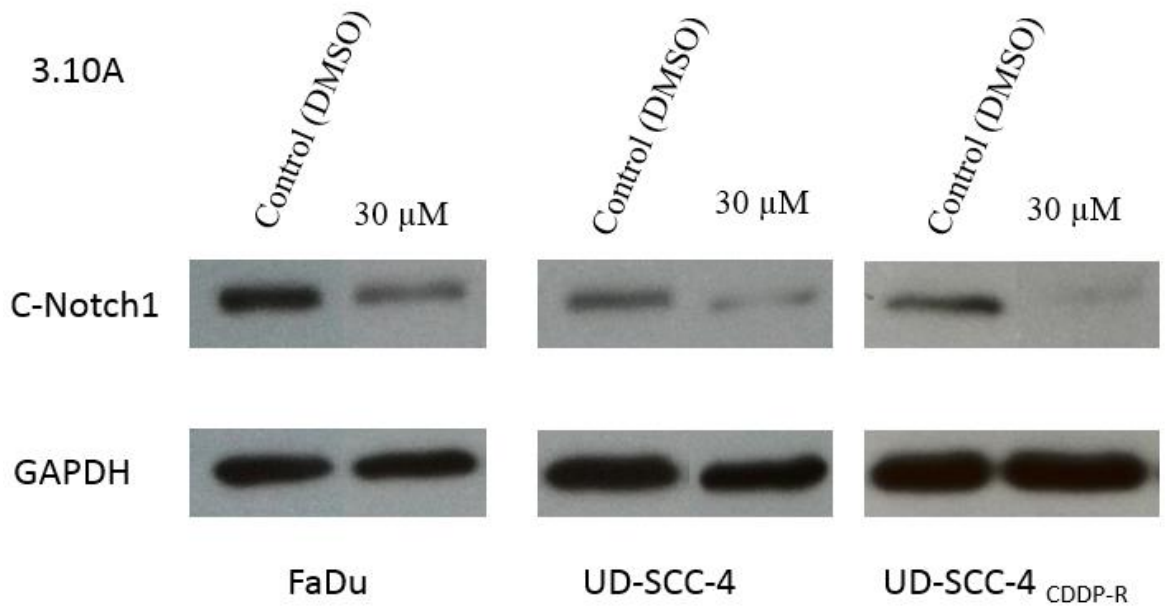


Figure 3.9: C-Notch1 protein levels in FaDu $_{CDDP-R}$ cells 72 hours after treatment with different concentrations of GSIs. A) Western blot analysis was performed to examine the status of activated NICD. B) Quantification of C-Notch1 protein levels (relative expression to GAPDH protein levels) for different concentrations of GSIs are shown.

Furthermore, western blot analysis was performed to assess the effects of GSI IX on C-Notch1 expression in the other cell lines. As shown in Figure 3.10, compared with control group, lower levels of C-Notch1 were detected in cells treated with GSI IX (30 µM). Quantification of relative C-Notch1 protein levels (normalized to GAPDH protein levels) showed that reduction rates of C-Notch1 protein for FaDu, UD-SCC4 and UD-SCC-4 $_{CDDP-R}$ were 36%, 20% and 26%, respectively (Figure 3.10B). The extent of reduction was slightly higher in cisplatin-resistant cells than in cisplatin-sensitive cells.



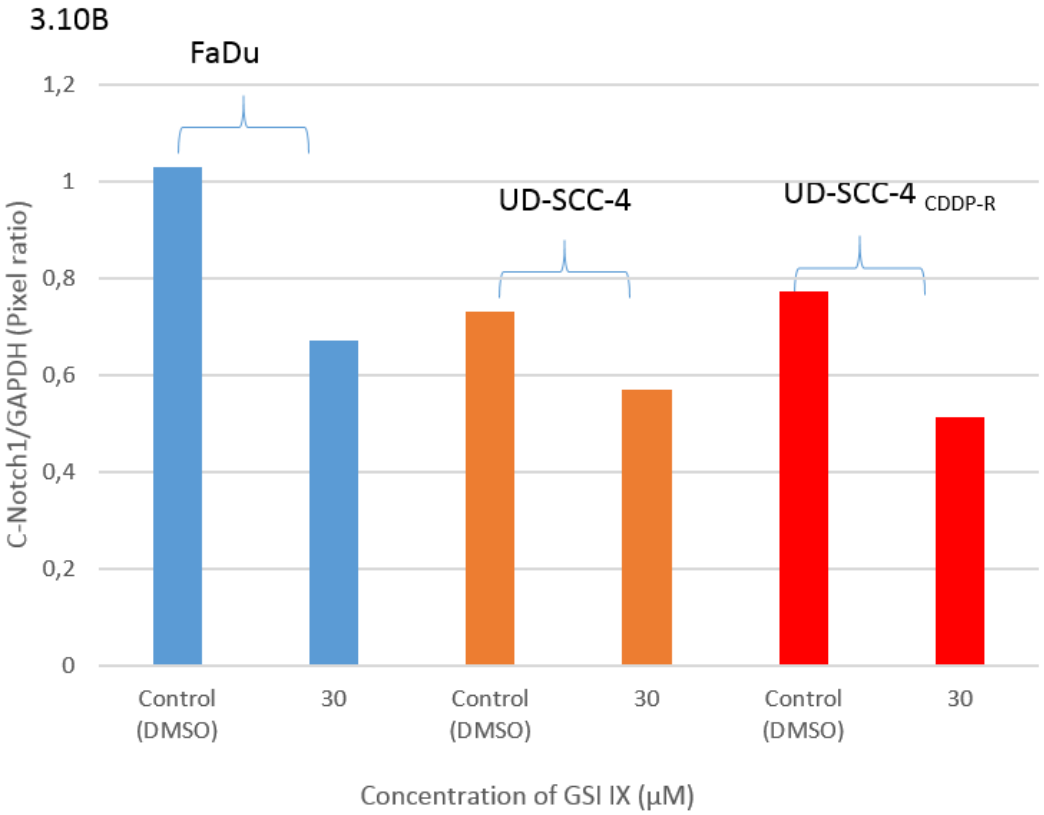
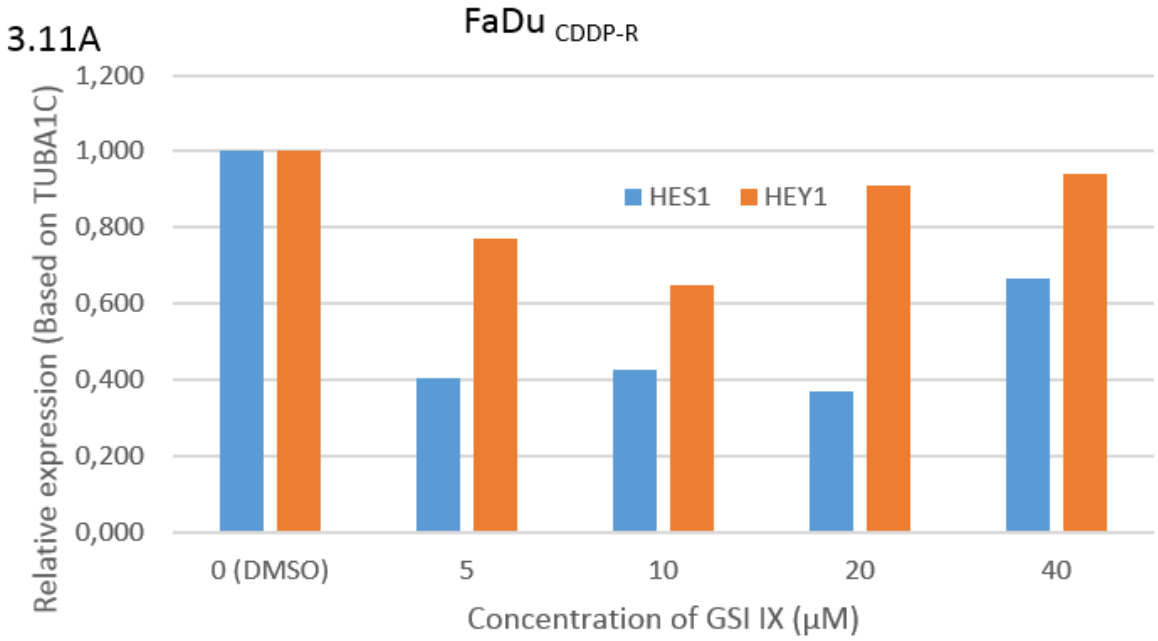


Figure 3.10: C-Notch1 protein levels were determined in FaDu UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ cells 72 hours after treatment with 30 µM GSI IX. A) Western blot analysis was performed to examine the status of activated NICD. B) Quantification of C-Notch1 protein levels (relative expression to GAPDH protein levels) is shown.

3.8 Effect of GSI IX treatment on the expression of Notch1 target genes

In order to further validate our previous results, the extent of inhibition of Notch1 and its downstream signaling pathway by GSI IX, the inhibitor of γ-secretase complex was determined. For this purpose, the expression of Notch1 target genes (Hes1 and Hey1) after GSI IX treatment was evaluated. Real-time RT-PCR was performed in UD-SCC-4 $_{CDDP-R}$ and FaDu $_{CDDP-R}$ cells, in which C-Notch1 expression was shown to be inhibited by GSI IX (Figure 3.9 and 3.10). Cells were treated with different concentrations of GSI IX for 72 hours in a 6-well plate. Control cells were treated with DMSO. Afterwards, total RNA was extracted and real-time RT-PCR was performed. As shown in Figure 3.11A, Hes1 expression was suppressed by 60% in FaDu $_{CDDP-R}$ and by 50% UD-SCC-4 $_{CDDP-R}$ after inhibition of C-Notch1. Similarly, Hey1 was suppressed by 60% in UD-SCC-4 $_{CDDP-R}$, shown in Figure 3.11B. However, compared with the DMSO group, GSI IX treated FaDu $_{CDDP-R}$ cells did not showed a remarkably reduced expression of Hey1 (Figure 10A). Altogether, we demonstrated that Hes1 and Hey1 can be reduced by GSI IX treatment, but the inhibitory effect depended on the cell line and concentration of GSI IX.



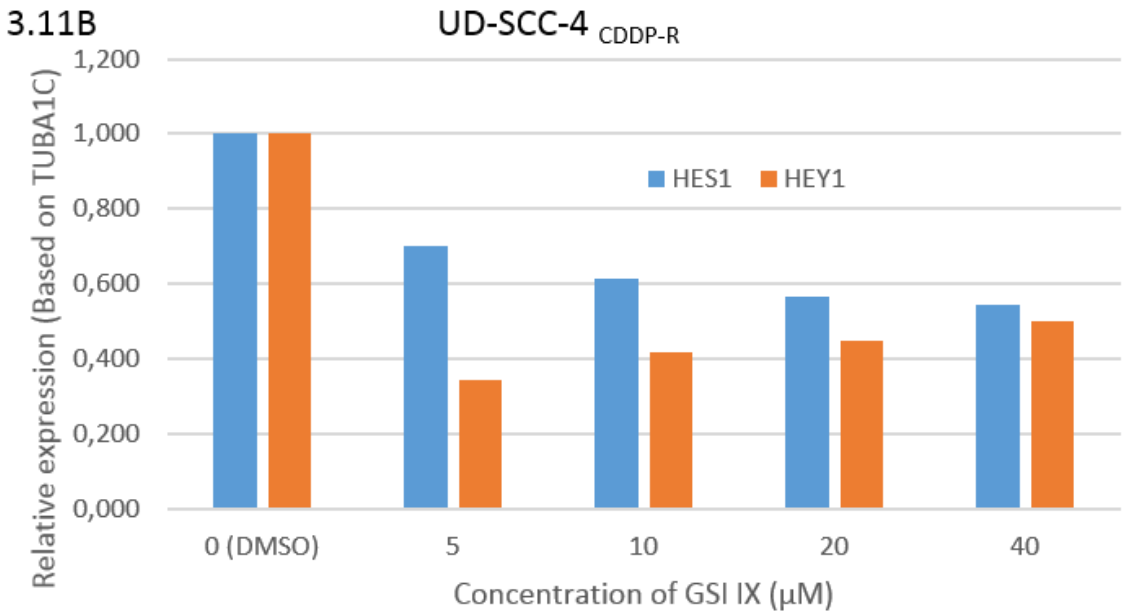


Figure 3.11: Hes1 and Hey1 expression after inhibition of Notch inactivation by GSI IX treatment in FaDu $_{CDDP-R}$ (A) and UD-SCC-4 $_{CDDP-R}$ cells (B).

3.9 Effect of GSI IX treatment on cisplatin sensitivity

Since Hes1 and Hey1 expression could be reduced after inhibition of Notch activation by GSI IX treatment, I next assessed whether GSI IX was able to sensitize HNSCC cells to cisplatin treatment. Cells were cultured in 96-well plates and treated with 30 µM GSI IX for 24 hours before starting treatment with cisplatin. As read-out for cisplatin sensitivity, cell viability was assessed by MTT assays. Unexpectedly, GSI IX pretreatment reduced rather than increased sensitivity of HNSCC cells to cisplatin. The IC50 for cisplatin in the control group was significantly lower than in the GSI IX treated group in the four HNSCC cell lines. This trend became more apparent with increasing cisplatin concentrations (Figure 3.12). Moreover, there was no difference in the effect of GSI IX treatment between resistant cells and their sensitive parental cells. IC50 and DRI were also calculated (Table 3.4). Taken together, these results suggested that inhibition of Notch1 signaling by GSI IX treatment renders HNSCC cells less sensitive to cisplatin treatment.

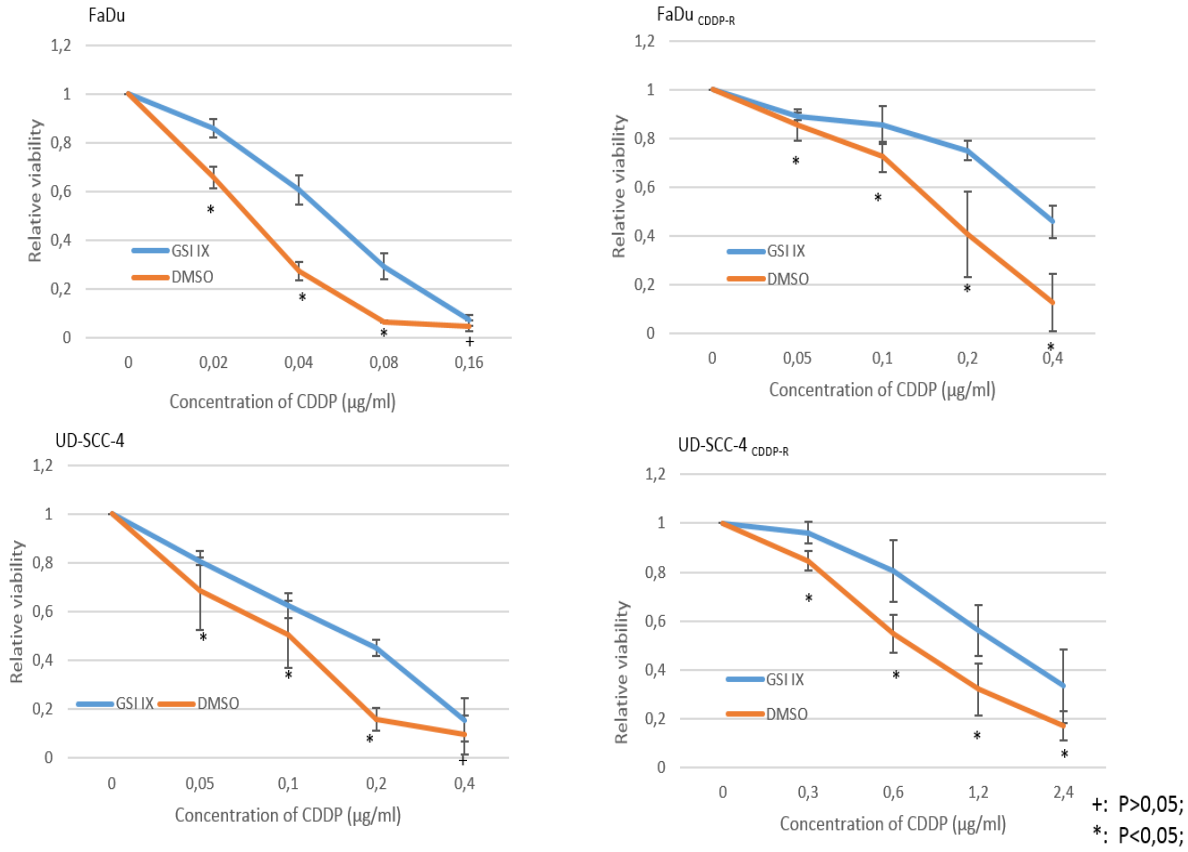


Figure 3.12: Effects of Notch1 inhibition by GSI IX treatment on cisplatin sensitivity in four HNSCC cell lines. Cell viability was assessed by MTT assay by measuring the optical densities (OD) at 540 nm. Results of at least three independent experiments are shown.

Table 3.4 IC50 and DRI of cisplatin in HNSCC cells treated with GSI IX (mean ± SD)

	IC50 of cisplatin (µg/ml)		DRI	P value
	GSI IX	Control (DMSO)		
FaDu	0.050±0.007	0.026±0.001	1.923	0.003
FaDu $_{CDDP-R}$	0.407±0.107	0.163±0.022	2.500	0.009
UD-SCC-4	0.149±0.026	0.085±0.026	1.753	0.034
UD-SCC-4 $_{CDDP-R}$	1.552±0.342	0.773±0.178	2.008	0.018

To better understand the effect of GSI IX on cisplatin sensitivity in HNSCC cells, we studied changes in cell morphology and cell numbers. Again, cells were treated with 30 µM GSI IX in 6-well plates for 24 hours before cisplatin treatment to reduce NICD1 expression. The appropriate cisplatin concentrations necessary for killing 40-50% cells for FaDu, FaDu $_{CDDP-R}$, UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ were 2 µg/ml, 4 µg/ml, 3 µg/ml and 10 µg/ml, respectively. As shown in Figure 3.13, untreated (3.13A) and DMSO treated cultures (3.13B) of UD-SCC-4 cells showed the highest cell density, and apoptotic cells accounted for a relatively low proportion. There was no difference of cell density and morphology between cells treated with GSI IX at a dose of 30 µM (3.13C) and untreated control cells. In addition, no effect on cell adhesion was demonstrated. Significant changes in morphology and cell density occurred in cell cultures at 48 hours after cisplatin treatment (3.13E). The number of adherent cells decreased and the proportion of apoptotic cells and cell debris increased. However, when cells exposed to GSI IX 24 hours before cisplatin treatment, more cells survived and less apoptotic cells were observed (3.13D). Similar results were obtained in the other three cell lines.

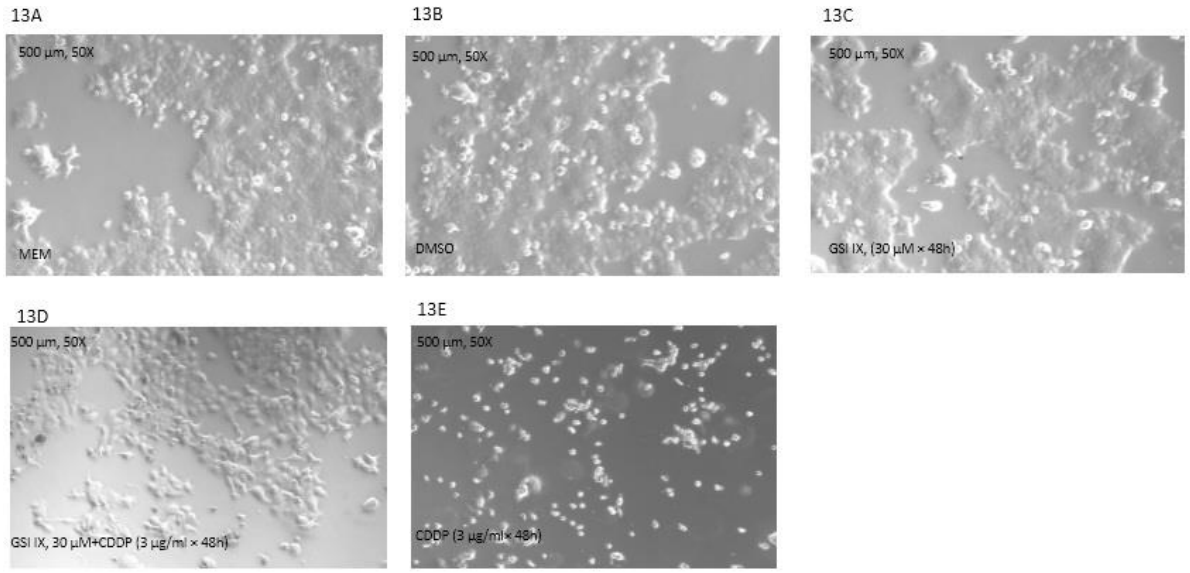
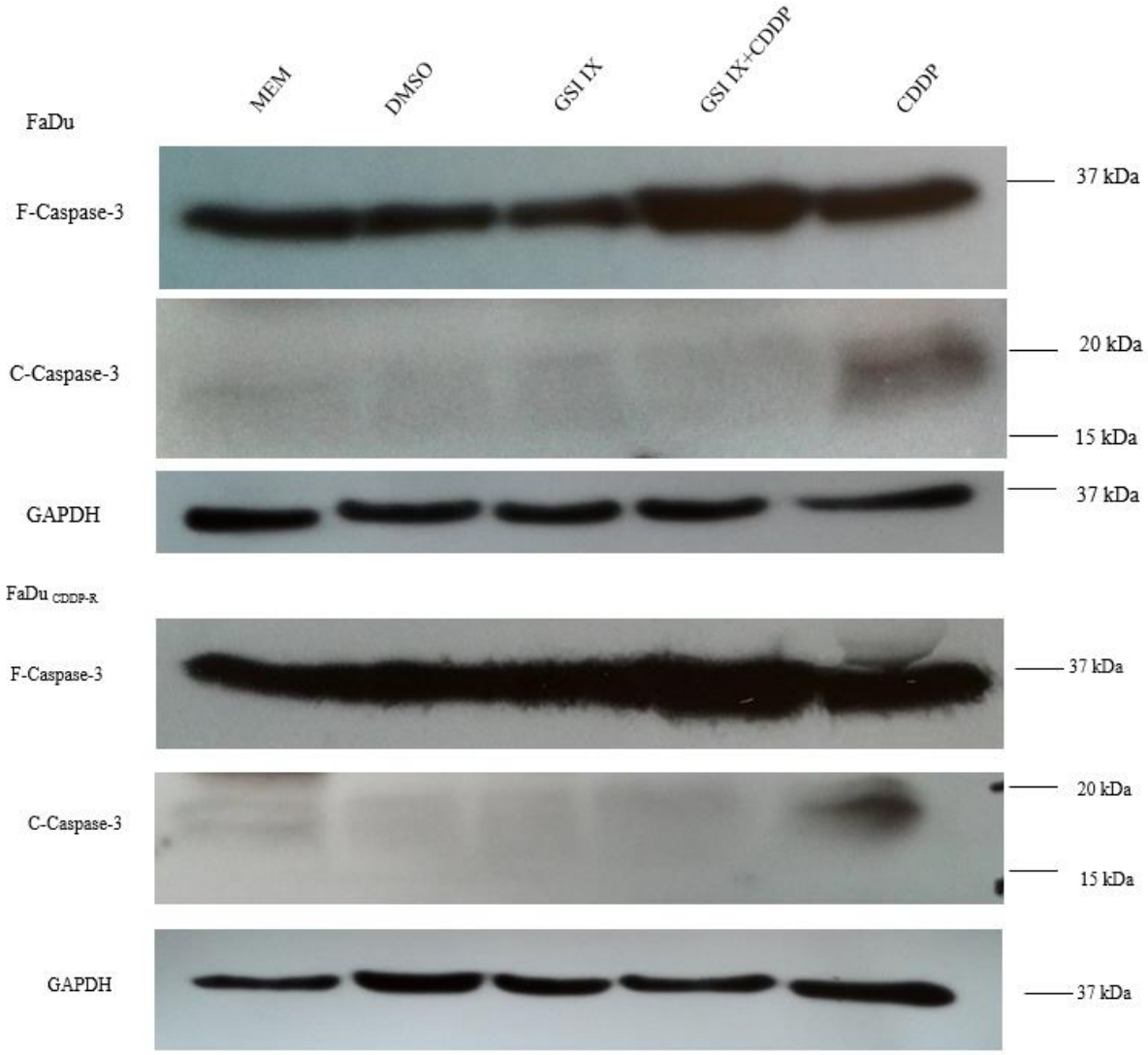


Figure 3.13: Effect of CDDP with or without GSI IX on UD-SCC-4 cells. Cells were observed and pictures were taken under the light microscope (original magnification 50×).

Caspase family plays a crucial role in the regulation of cell apoptosis. It is generally appreciated that caspase-3 is one of the most important apoptosis executioners. Our MTT results demonstrated that pretreatment with GSI IX decreased the cytotoxic effect of cisplatin in HNSCC cells. To better understand the effect of GSI IX on cisplatin sensitivity in HNSCC, we examined the expression of caspase-3 (F-Caspase-3 or Pro-caspase-3) and cleaved caspase-3 (C-Caspase-3 or Active caspase-3) in FaDu, UD-SCC-4 and their resistant subclones by immunoblotting. In 6-well plates, FaDu, FaDu $_{CDDP-R}$, UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ were treated with cisplatin at doses of 2 µg/ml, 4 µg/ml, 3 µg/ml and 10 µg/ml, respectively. Again, more cells survived when cells were treated with GSI IX at a dose of 30 µM for 24 hours before cisplatin treatment. Forty-eight hours after cisplatin treatment, proteins were extracted from cell lysates and subjected for western blot. As shown in Figure 3.14, cisplatin treatment increased the levels of activated caspase-3 in cell lines, compared to untreated or DMSO-treated cells. However, when cells were pretreated with GSI IX, expression of activated caspase-3 was attenuated. Furthermore, quantitative analysis of the western blot results confirmed the significant difference in expression levels of activated caspase-3 between cisplatin-treated cells and cells treated with cisplatin in combination with GSI IX (Figure 3.15). Thus, the reduction of cisplatin sensitivity by GSI IX seems to be mediated at least in part by its inhibition of caspase-3-dependent apoptosis.


MEM
DMSO
GSI IX
GSI IX+CDDP
CDDP
FaDu
F-Caspase-3
37 kDa
C-Caspase-3
20 kDa
15 kDa
GAPDH
37 kDa
FaDu CDDP-R
F-Caspase-3
37 kDa
C-Caspase-3
20 kDa
15 kDa
GAPDH
37 kDa

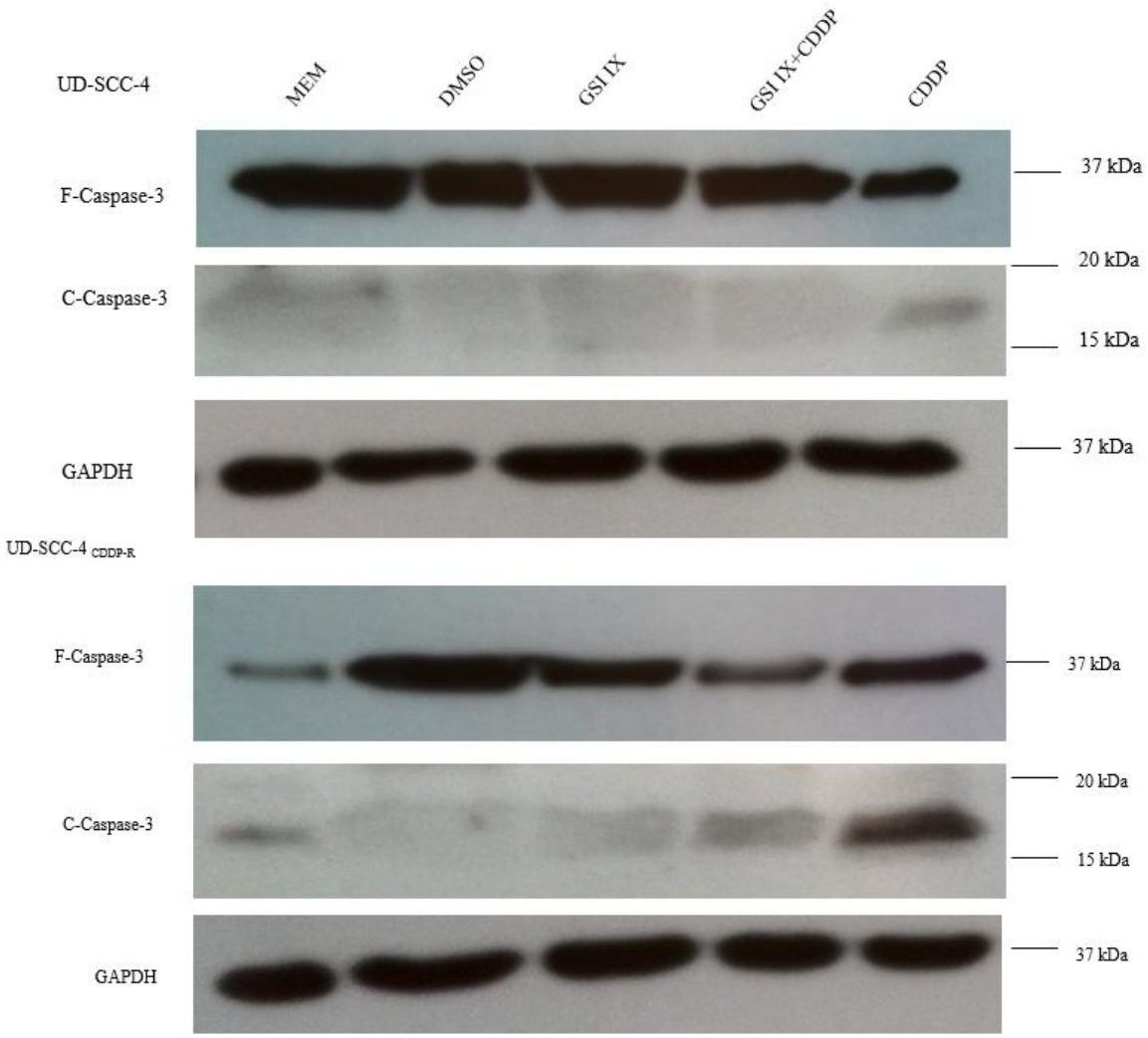


Figure 3.14: Effect of cisplatin with or without GSI IX on the expression of active caspase-3 in HNSCC cells.

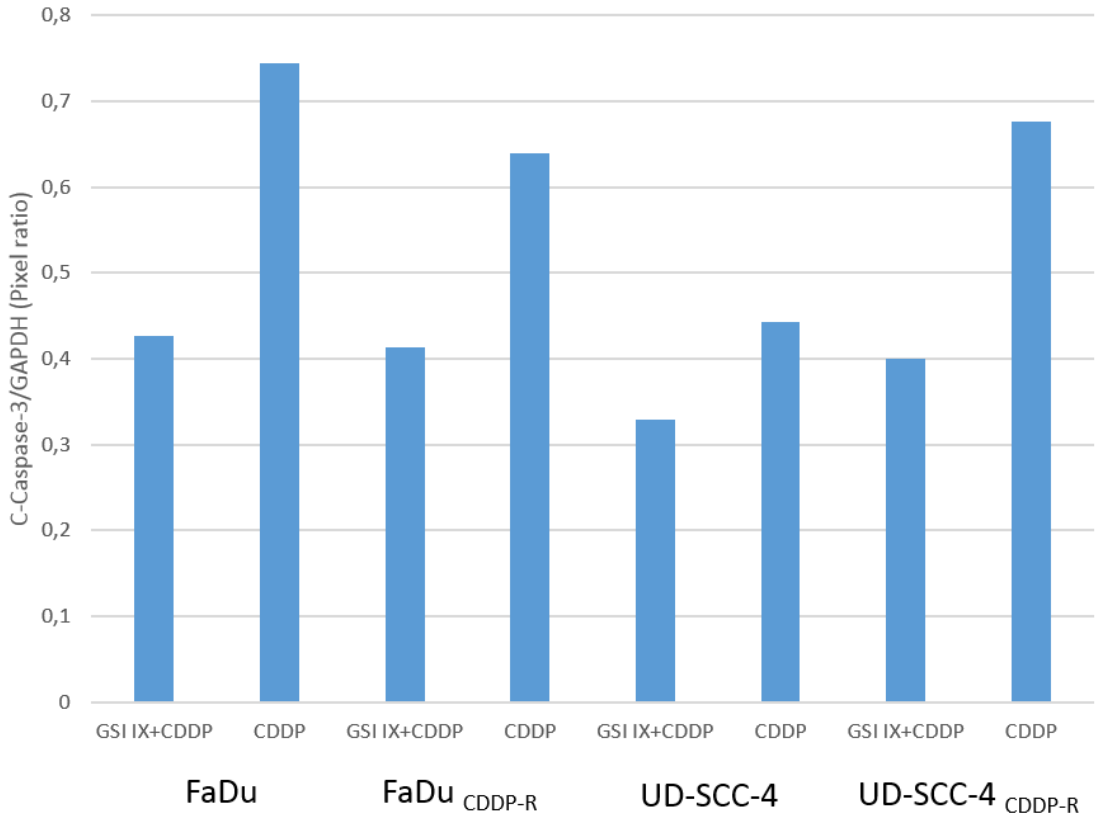


Figure 3.15: Quantification of C-caspase-3 protein levels (relative expression to GAPDH protein levels) was shown.

3.10 Effect of GSI IX on radiation sensitivity of HNSCC cells

Next, it was determined whether GSI IX also affects the radiation sensitivity of HNSCC cell lines. Cells were cultured in 96 well plates and were left untreated or were treated with GSI IX at concentration of 30 µM for 24 hours before irradiation. Again, the radiation dose for all cell lines were 0 to 6 Gy. After culture of cells for six to eight days, MTT assays were performed. The relative viability of the cells in each group was determined and dose response curves were calculated. Slight differences in survival rates between GSI IX treated cells and control cells were observed (Figure 3.16). However, this difference did not reach statistical significance in FaDu $_{CDDP-R}$, UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$ (all $p>0.05$). In FaDu cells, cell survival at 2 Gy or 4 Gy increased by 10% if cells were pretreated with GSI IX (2 Gy, $p=0.018$; 4 Gy, $p=0.005$). There was no significant difference in calculated IC50 values for radiation between FaDu and UD-SCC-4 cell lines and their resistant cells (all $p > 0.05$). In summary, the data speak against a major effect of GSI IX on the sensitivity of HNSCC cells to radiation.

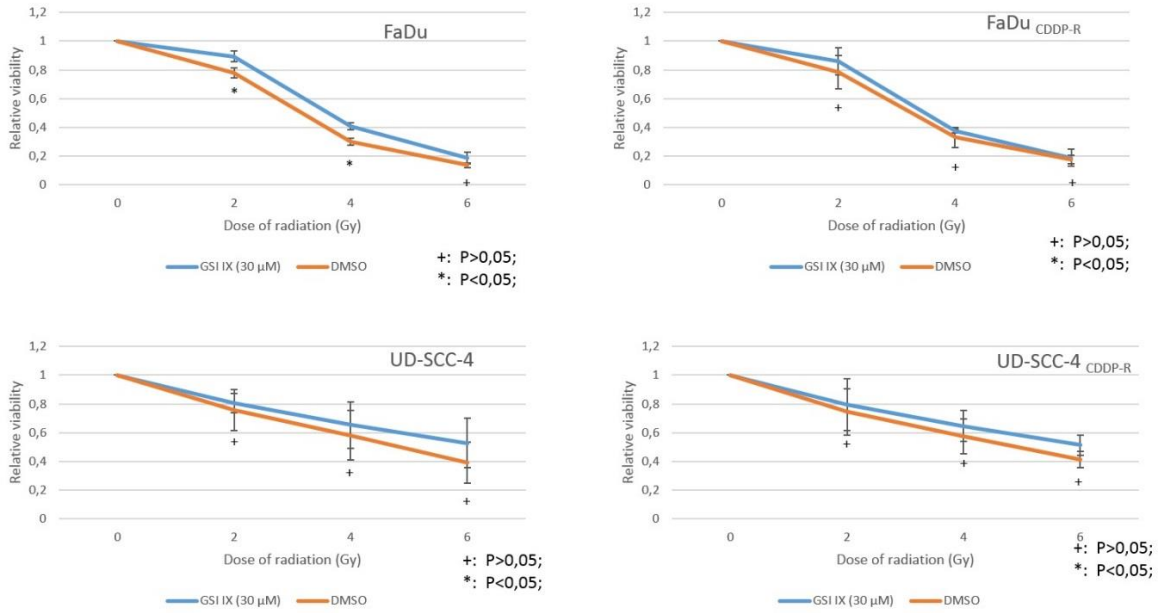


Figure 3.16: Effects of GSI IX on radiation sensitivity in four HNSCC cell lines. The proliferation rate of the cells in each group was determined by long-term MTT assay. Results for three independent experiments are shown.

Table 3.5 Determination of IC50 and resistance index of radiation in HNSCC cells treated with GSI IX and DMSO (mean ± SD)

Cell line	IC50 of radiation (Gy)		RI	p
	GSI IX	DMSO		
FaDu	3.55±0.19	3.06±0.15	1.16	0.068
FaDu $_{CDDP-R}$	3.54±0.38	3.20±0.51	1.11	0.407
UD-SCC-4	6.20±1.85	4.74±1.62	1.31	0.267
UD-SCC-4 $_{CDDP-R}$	6.13±0.80	4.92±1.81	1.25	0.350

3.11 Relative mRNA expression levels of Notch family members in HNSCC cell lines

As shown above, GSI IX pretreatment decreased the sensitivity of HNSCC cells to cisplatin whereas siRNA-mediated knock down of Notch1 failed to do so. Since it is known that γ-secretase inhibitors have many targets beside Notch1, I hypothesized that GSI IX altered cisplatin sensitivity not by inhibition of Notch1 but by targeting another Notch protein. Therefore, I also measured the gene expression of the other Notch genes, Notch2-4, in the four HNSCC cell lines. As shown in Figure 3.17, the UD-SCC-4 $_{CDDP-R}$ expressed highest levels of Notch3. In general, the HNSCC

cells except FaDu expressed significantly elevated levels of Notch3, compared to Notch1, Notch2 and Notch4. In FaDu cell line, similar expression patterns were observed for Notch2 and Notch3. Because most prominent differences between sensitive and resistant cell lines were determined for Notch3 expression (Shown in Figure 3.17), we hypothesize that GSI IX might change cisplatin sensitivity by blocking the activation of Notch3.

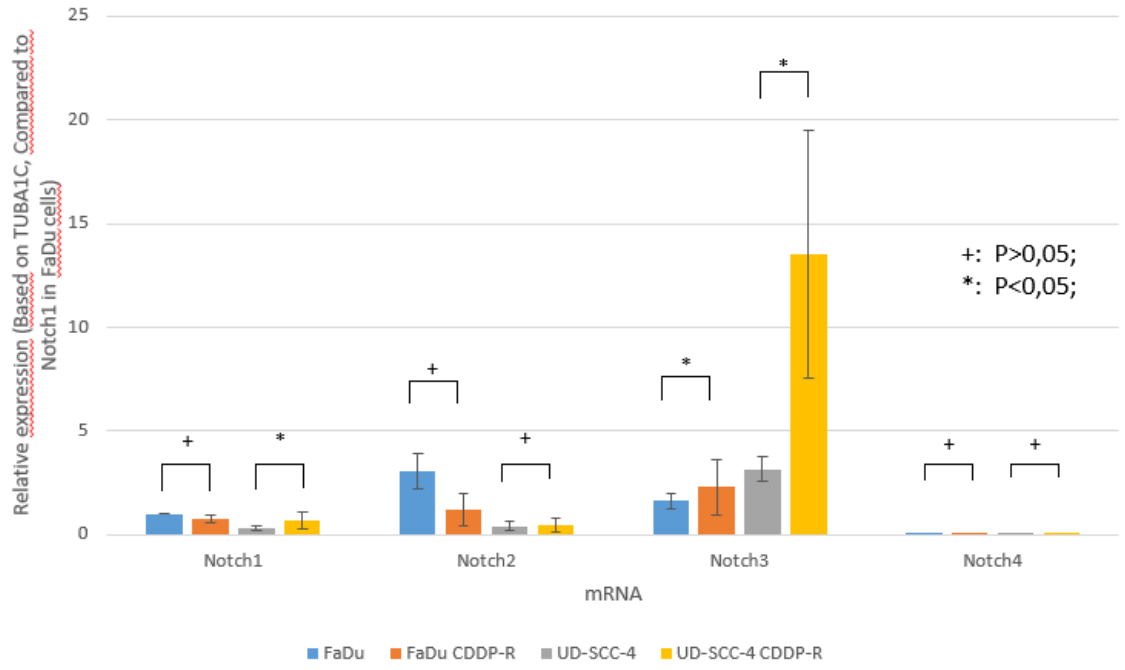


Figure 3.17: Notch1, Notch2, Notch3 and Notch4 relative mRNA expression (based on TUBA1C) were evaluated by real-time RT-PCR. Data were normalized to the value of Notch1 mRNA levels in FaDu cells. Columns and cross bars represent the average and standard deviation of three biological replicates respectively, each from a different passage of cells. The expression levels of Notch4 were very low in all cell lines.

3.12 Establishment of Notch3 knock down by transfection with Notch3 siRNA

In the next step it was investigated whether knock down of Notch3 could change cisplatin sensitivity in HNSCC cells. The objective of this part of project was to evaluate the effect of Notch3 siRNA transfection on Notch3 protein and mRNA expression in HNSCC cell lines. Another aim was to find the optimal siRNA concentration and time point after transfection for subsequent experiments.

UD-SCC-4 $_{CDDP-R}$ showed highest mRNA relative expression of Notch3 in the four cell lines (Figure 3.17). Therefore, UD-SCC-4 $_{CDDP-R}$ was selected for the establishment of Notch3 siRNA transfection. UD-SCC-4 $_{CDDP-R}$ were transfected with different concentrations of Notch3 siRNA

(10 nM, 25 nM and 50 nM) or non-target siRNA (NTC, 25 nM) in a 6-well plate and harvested 48 or 72 hours after transfection. As shown in Figure 3.18, UD-SCC-4 $_{CDDP-R}$ cells transfected with Notch3 siRNA displayed a reduction in the protein levels of C-Notch3 by western blot analysis. Additionally, knock down of full length Notch3 protein was demonstrated in cells 72 hours after transfection. Non-target siRNA did not affect Notch3 expression. Furthermore, the suppression of Notch3 by siRNA transfection in cells was confirmed by real-time RT-PCR analysis. Therefore, UD-SCC-4 cells were harvested from a 6-well plate 72 h after transfection and subjected for RNA isolation and cDNA synthesis. Quantitative analysis of mRNA expression by real-time PCR showed that the efficiency of Notch3 gene silencing was 75% in FaDu, about >80% in FaDu $_{CDDP-R}$, 92% in UD-SCC-4 and 83% in UD-SCC-4 $_{CDDP-R}$ at 48 hours post-transfection, compared to NTC siRNA (Figure 3.19). Based on these findings, we selected the dose of 25 nM for knockdown and the optimal timepoint for harvesting as 48 h after transfection.

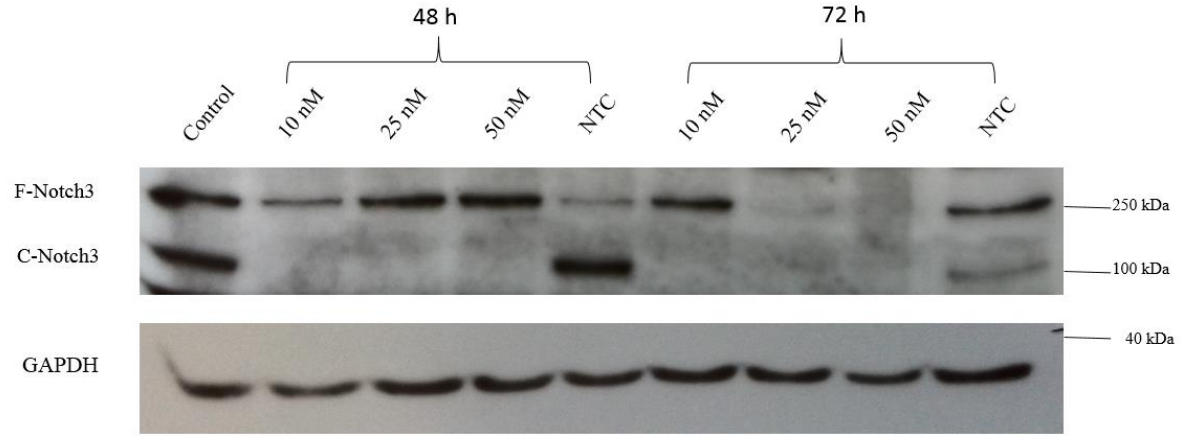


Figure 3.18: Downregulation of Notch3 expression following Notch3 siRNA transfection.

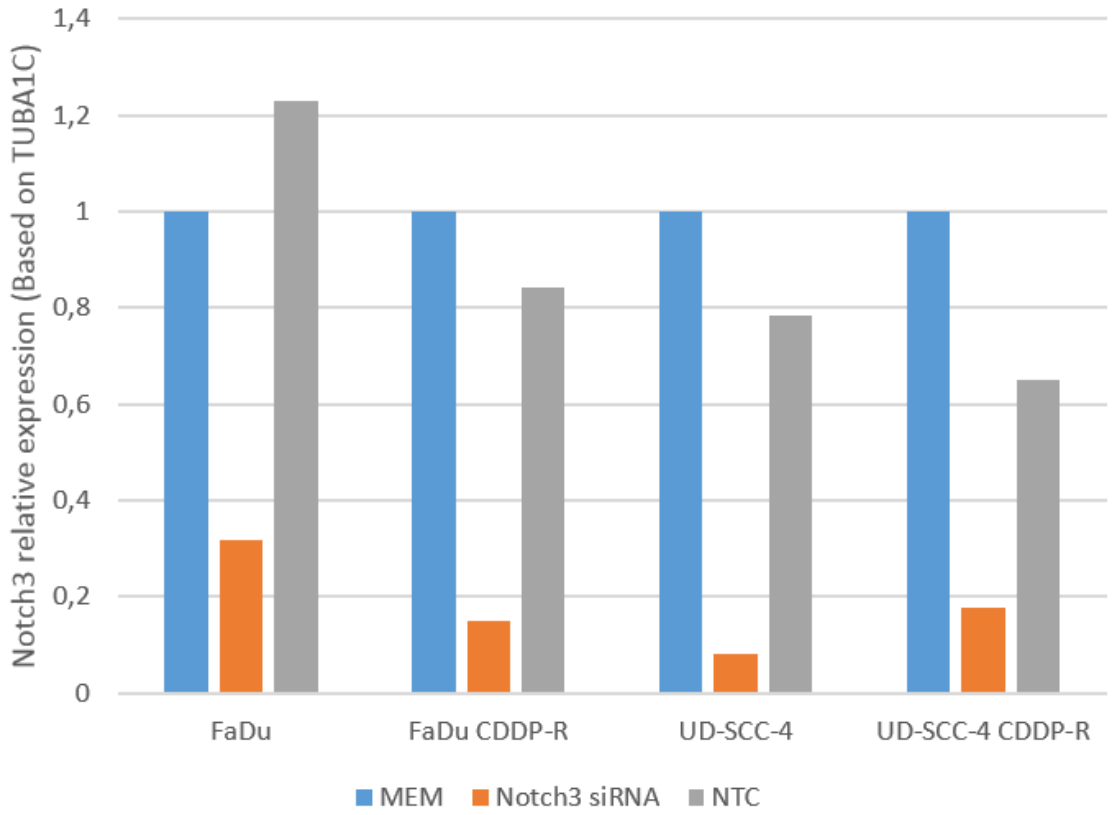


Figure 3.19: Effect of Notch3 siRNA transfection was confirmed by real-time RT-PCR in FaDu and UD- SCC-4 cells and their corresponding resistant cells.

3.13 Effect of Notch3 siRNA transfection on the expression of its target genes

As Notch3 expression was decreased by Notch3 siRNA transfection, the expression of its target genes Hes1 and Hey1 after Notch3 siRNA transfection was also evaluated. Real-time RT-PCR was performed in the four HNSCC cell lines according to the established protocol. Following the transfection with siRNA, total RNA was extracted from cells 48 h after transfection and subjected to real-time RT-PCR. Compared to the NTC siRNA group, Notch3 knock down did not result in a reduction in the expression of Hes1 and Hey1 in FaDu cells and only slightly decreased the expression levels of both genes in UD-SCC-4 cells (Figure 3.20).

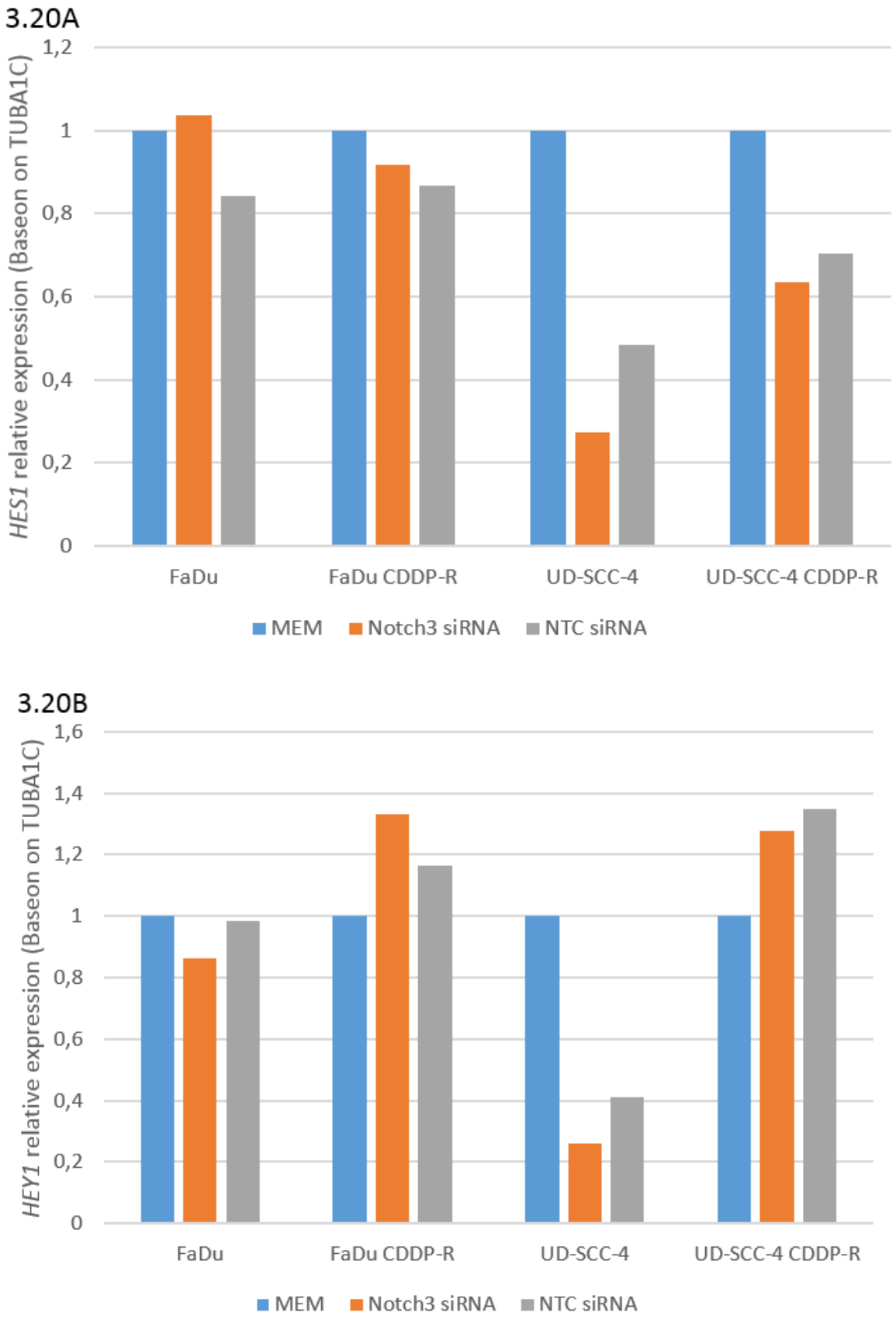


Figure 3.20: The changes of Hes1 (A) and Hey1 (B) 48 hours post Notch3 siRNA transfection, determined by real-time RT-PCR. *TUBA1C* expression levels were used for normalization.

3.14 Effect of Notch3 knockdown on cisplatin sensitivity

Since the Notch3 siRNA transfection reduced expression of full-length as well as cleaved Notch3 protein, I determined whether knock down of activated Notch3 would affect the cisplatin sensitivity of HNSCC cell lines. Long term culture and subsequent MTT assay were used to compare the cisplatin sensitivity of cells treated with the Notch3 siRNA or NTC siRNA. Because the results of real-time RT-PCR demonstrated a slightly decreased expression of the Notch target genes (*HES1* and *HEY1*) by NTC siRNA transfection as well, more controls were included in the analysis to determine potential unspecific effects by the siRNA transfection procedure itself. Therefore, cells were divided into four groups: cells which were left untreated (MEM group) and those which were treated with the transfection reagent alone, combined with Notch3 siRNA or NTC siRNA. As shown in Figure 3.21, dose-response curves from MTT assays demonstrated no difference in survival rates between these four groups in the four cell lines ($p>0.05$). The IC50 values and RI were calculated in these four cell lines (Table 3.6, $p>0.05$). As already shown for Notch1, these data suggest that siRNA-targeting of Notch3 does not interfere with the sensitivity of HNSCC cells to cisplatin.

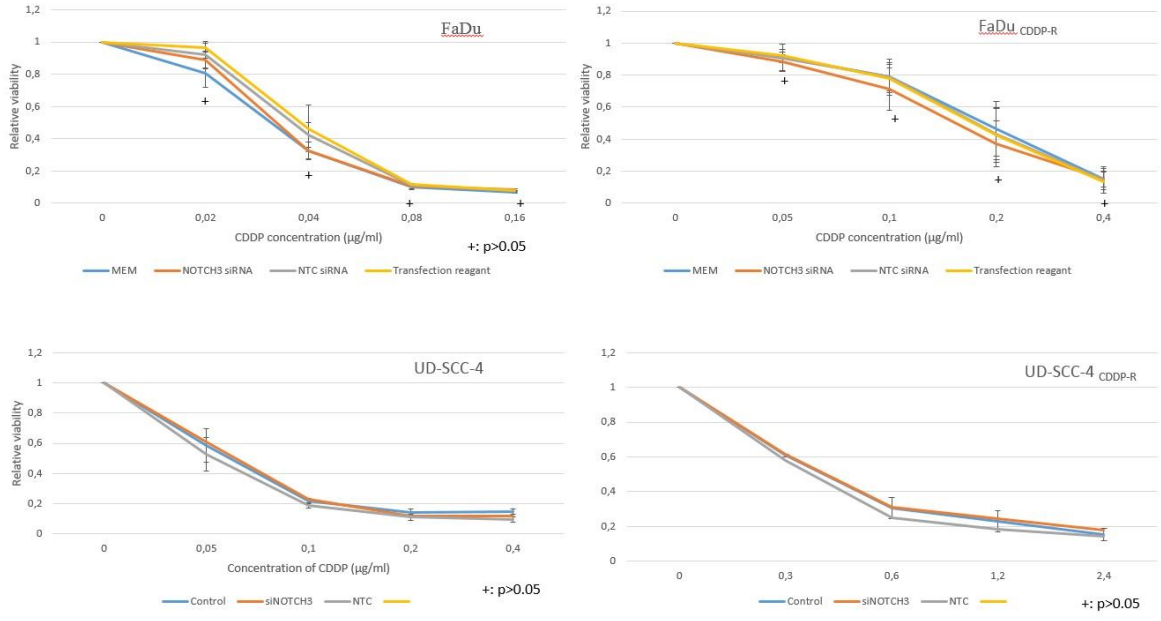


Figure 3.21: Effects of Notch3 knock down on cisplatin sensitivity in four HNSCC cell lines. MTT assays were performed after siRNA mediated knock down of Notch3 and long term culture with increasing concentrations of cisplatin for six days. Results are shown for three independent experiments.

Table 3.6 Determination of IC50 and RI of cisplatin in HNSCC cells treated with Notch3 siRNA (mean ± SD)

	IC50 of cisplatin (μg/ml)				RI	P
	MEM	Notch3 siRNA	NTC siRNA	Transfection reagent		
FaDu	0.033±0.006	0.036±0.003	0.040±0.003	0.043±0.006	0.900	0.289
FaDu $_{CDDP-R}$	0.188±0.043	0.158±0.044	0.180±0.051	0.176±0.046	1.190	0.904
UD-SCC-4	0.051±0.001	0.055±0.022	0.044±0.014	-	1.250	0.610
UD-SCC-4 $_{CDDP-R}$	0.411±0.090	0.373±0.075	0.301±0.032	-	1.239	0.354

4. Discussion

Cisplatin has maintained widespread clinical use in solid tumors, including HNSCC. Intrinsic or acquired resistance to cisplatin remains a major hindrance for patients with HNSCC. However, the mechanisms causing cisplatin resistance are still poorly understood and no previous trials have been successful in determining a regimen which could increase cisplatin sensitivity. Recently, many evidences suggested that Notch signaling pathway might be related to cisplatin sensitivity [90, 96, 97]. Therefore, the current study was conducted to prove this hypothesis.

4.1 Cisplatin resistance and radio-sensitivity in the resistant models of HNSCC cell lines

In our lab, three models of HNSCC cell lines which had acquired resistance to cisplatin (FaDu, UD-SCC-4 and UM-SCC-22B) were already established by long-term treatment of cisplatin [98]. Two of them, FaDu and UD-SCC-4, demonstrated wildtype status of the *NOTCH1* gene in previous NGS analyses for both sensitive and resistant cells. In contrast, a protein truncating nonsense mutation could be identified in the sensitive and resistant cell line UM-SCC-22B serving as a knockout model for Notch1.

Cisplatin-based concurrent chemo-radiotherapy is now considered to be the well-established standard treatment for patients with HNSCC. Overcoming radio-resistance may provide opportunity to cure HNSCC. Therefore, we also investigated radio-resistance in these models. In the literature contradictory results have been reported: In several publications, tumor cells from glioma, lung or ovarian cancer showed not only a cisplatin-resistant phenotype but were also cross-resistant to radiation [99-103]. We also evaluated the IC50 of radiation in the four HNSCC cell lines. Long-term cisplatin treatment of FaDu and UD-SCC-4 only induced cisplatin resistance, but did not increase the cross-resistance to radiation. This is consistent with findings from Wallner KE et al. [104], demonstrating comparable efficacy in the repair of sub-lethal or potentially lethal radiation damage in cisplatin-resistant and -sensitive cells. Resistance to cisplatin could be a result of decreased drug uptake, increased efflux or increased inactivation by sulfhydryl molecules such as glutathione. Cross resistance of tumors between cisplatin and radiation may be a result of the tumor microenvironment, rather than being a cellular phenomenon [104], resulting from decreased tumor blood supply [105] or reduced cellular metabolism [106]. In contrast, resistance to radiation is thought to be mainly caused by tumor hypoxia. That means in HNSCC patients for whom concurrent chemotherapy is not available, we could use cisplatin as a neoadjuvant chemotherapy because the effect of subsequent radiotherapy will not be influenced. The clinical relevance of our findings needs to be further investigated.

4.2 Notch1 expression in HNSCC cell lines

In our study, Notch1 expression both at protein level and mRNA level were higher in FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ compared to their isogenic sensitive parental cells (FaDu and UD-SCC-4), indicating that Notch1 might indeed be involved in cisplatin resistance in HNSCC. Overexpression of Notch1 could increase its positive effects on c-myc activity [90, 107], via interacting with α-enolase [107], which has been described as a potential cisplatin resistance factor [108]. Hence, an overexpression of Notch1 might also endorse HNSCC cells with high stemness, more chemotherapy resistance and stronger invasion potential [109]. These results would support the hypothesis that Notch1 expression is positively correlated to cisplatin resistance. Although it needs more studies to prove this hypothesis, especially in clinical settings, it has long been anticipated that Notch1 might be used as an effective biomarker for cisplatin chemotherapy in HNSCC treatment. Detection of Notch1 expression could allow clinicians to choose the appropriate anticancer agents in treatment. However, these findings are in contrast to other studies in which tumor suppressor properties of Notch1 have been described.

Not only were there several studies demonstrating Notch1 as a tumor suppressor in HNSCC, but also the results from the current study show that double-edge function of Notch1 might exists. The results from western blot and real-time RT-PCR showed that although Notch1 expression in was higher FaDu than in UD-SCC-4 cells, increased cytotoxicity of cisplatin was observe in the former model. The possible reason to explain why the results from the comparative analysis of the cisplatin-sensitive and -resistant subclones of syngenic HNSCC cell line models support an oncogenic role of Notch1 whereas the comparison of genetically different cell lines rather speak for a tumor suppressor role is these two cell line models derived from different localizations of head and neck. FaDu cells were derived from hypopharynx and UD-SCC-4 cells were derived from oropharynx. There might be different changes on the genetic level, which result in the overexpression of Notch1. Therefore, detailed comparisons of genetic alterations in HNSCC cells containing activating Notch1 pathway, together with functional studies *in vitro* and *in vivo*, are required to explain the contradicting role of Notch1 in HNSCC.

Our study confirmed that HNSCC cells are characterized by positive expression pattern of F-Notch1 protein and active Notch1 protein. A significant association was observed between mRNA level and protein expression only in UD-SCC-4 and UD-SCC-4 $_{CDDP-R}$. In contrast, in FaDu and its cisplatin-resistant derivative, Notch1 mRNA levels were not correlated with protein expression, which is consistent with a previous study in laryngeal cancer [110]. In contrast, previous evidence

from clinical cohort studies has been provided linking Notch1 overexpression, both at the protein and mRNA level, to advanced disease and poor outcome especially in tongue cancer [86, 88]. This discrepancy on the prognostic role of mRNA vs. protein suggests that Notch1 functions in a highly context-dependent manner acting differently in diverse tissues [110]. Nevertheless, Notch1 protein plays a critical role in acquired cisplatin resistance in HNSCC cells. Furthermore, whether both Notch1 protein and mRNA expression could be used as prognostic factors in all subtypes of HNSCC needs further validation.

4.3 Silencing of Notch1 did not change the sensitivity to cisplatin in HNSCC

We have confirmed the Notch1 was higher expression in FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$, compared to their cisplatin sensitive cells. Therefore, we assumed that a reduction of Notch1 protein levels will alter the cisplatin sensitivity in the HNSCC cells. Surprisingly, the results from MTT assay indicated that there was no significant alteration in cisplatin sensitivity after knockdown of Notch1 in all cell lines, in contrast to a recent report on HNSCC. In the study of Gu et al. [90], they examined expression of Notch1 in tumor tissues of 25 HNSCC patients. Meanwhile, cisplatin sensitivity was examined in cells isolated from the same tumor tissues. The obtained results showed high expression of Notch1 to be negatively correlated with cisplatin sensitivity in HNSCC. This is consistent with most evidences of cancer cells with activated Notch1 to be chemo-resistant [97, 111-113]. Active Notch1 signaling pathway could induce long noncoding RNA AK022798 expression, which participated in the process of cisplatin resistance [114] and reprogramming the chromatin state to promote cancer metastasis [115, 116]. Additionally, knockdown of Notch1 was demonstrated to suppress *ABCC2* and *ABCG2* transporter gene expression and decrease the expression of stem cell markers such as Oct4, Sox2, and CD44 [91]. This would reduce the efflux rate and stemness features of the tumor cells and overcome the chemo-resistance. Also, Notch1 was reported to relate with canonical Wnt signaling in HNSCC cells and Notch1 knockdown inhibited tumor formation and increased survival of mice in a xenograft model [91]. In conclusion, Notch1 may be a critical regulator of stemness in HNSCC cells, and knockdown of Notch1 could be a potential targeted approach for the treatment of HNSCC.

There were two possible reasons to explain the discrepancy between the result from our study and the results from the literature. First, the MTT assay measures the mitochondrial function and is one of the most frequently used method to detect cancer cell viability. The long-term MTT assay, providing a powerful tool to investigate toxicity *in vitro*, was a better method to measure cytostatic

changes caused by chemotherapy reagents, compared to short-term MTT assay [117]. The experiments performed in most of the previous studies were the short-term MTT assays (Shown in Table 4.1, 24-72 hours period), which could overestimate cytotoxicity by not accounting for reversible damage or regrowth of cancer cells resistant to chemotherapy reagents. The long-term MTT assay, with seven days of cisplatin incubation, could overcome this obstacle. In one early study [118], cell lines with a high proliferating cell rate (like cancer cell lines) showed a greater sensitivity to the drug in short-term MTT assay. However, in the long-term MTT assay no difference in sensitivity was observed. Furthermore, the unchanged cisplatin sensitivity of HNSCC cells upon knockdown of Notch1 also can be explained by the unaltered expression of Notch target genes (Hes1 and Hey1) after siRNA transfection. From result of real-time RT-PCR, siRNA inhibition of Notch1 on mRNA level was not complete and there might be some active Notch1 proteins left to compensate the effect. Moreover, we hypothesized that the expression levels of Hes1 and Hey1 were compensated after Notch1 knockdown by co-expression of other *NOTCH* genes in HNSCC cells. Therefore, we examined mRNA levels of all Notch receptors in four HNSCC cell lines and found Notch1 and Notch3 with the highest mRNA levels compared to Notch2 and Notch4. That is another reason why γ-secretase inhibitor, which is a pan-Notch inhibitor, was used in this project. γ-secretase inhibitor not only inhibited Notch1, but also other Notch proteins, which might induce the change in cisplatin sensitivity.

Table 4.1 Summary of studies demonstrated that knockdown of Notch1 influenced the sensitivity to chemotherapy reagents in cancer cells, using MTT assay

Refs	Cancer cell lines	Reagents	Effect on sensitivity	Treatment time	Implication
[91]	HNSCC	Cisplatin	Increase	72 hours	Stemness
[119]	Colorectal cancer	5-Fu Oxaliplatin	Increase	48 hours	Cell cycle
[120]	Breast cancer	Paclitaxel	Increase	24 hours	N. R
[121]	Prostate cancer	Docetaxel	Increase	24 hours	Apoptosis and cell cycle
[122]	Breast cancer	Docetaxel, Doxorubicin	Increase	48 hours	Apoptosis

Note: N.R: Not referred

4.4 Notch1 independent factors related with cisplatin resistance in HNSCC cells

The cisplatin-resistant derivative of the cell line UM-SCC-22B presented a *NOTCH1* nonsense mutation. It suggests that not only Notch1 is related to cisplatin resistance in HNSCC cells, but also other biological factors. The mechanism of cisplatin resistance is very complex. In a recent study [123], the parental UM-SCC-22B cell line was exposed to increasing concentrations of cisplatin from 0.5 to 12 μM during a three-month period. Finally, several corresponding resistant UM-SCC-22B cells, which were resistant to different concentrations of cisplatin, were obtained. The most resistance UM-SCC-22B $_{CDDP\text{-}R}$ formed more spheroids when cultured in ultralow attachment plates, compared to the parental UM-SCC-22B, indicating cisplatin-resistant cells are endowed with cancer stemness. Cisplatin resistant cells expressed more B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) and octamer-binding transcription factor 4 (OCT-4), two markers of stemness, than cisplatin sensitive cells. IL-6/STAT3 pathway was also found to be involved in cisplatin resistance in these models [123]. Therefore, the mechanism of cisplatin resistance among FaDu, UD-SCC-4 and UM-SCC-22B cells are expected to be significantly different. Understanding the mechanisms underlying resistance to cisplatin might have a profound impact in individual treatment in HNSCC.

4.5 Silencing of Notch1 did not change the sensitivity to radiation in HNSCC

Radiotherapy represents the most effective and important treatment of HNSCC, especially for locally advanced disease. However, there is less literature on the relationship between irradiation and Notch1 signaling pathway in HNSCC. Radiation has been shown to activate several Notch family member genes in several cancers [124, 125]. Activation of Notch1 signaling pathway and its ligands was observed rapidly after clinically relevant dose of irradiation, with a radiation dose-dependent pattern [125]. Inhibition of Notch1 led to a significant reduction in the size of cancer stem cell pool [125]. In another study a significant increase of Notch1 target gene expression, including *HES1, HEY1* and *HEY2*, 24 or 48 hours after irradiation, was observed in cutaneous T-cell lymphoma [126]. Few studies examined whether radio-sensitivity could be changed by silencing of Notch1. One prior study demonstrated that treatment of various human colorectal cancer cell lines with Notch1 siRNA significantly increased cell apoptosis induced by ionizing radiation [119]. The cytotoxic test used in this study was short-term MTT assay. But for detecting radio-sensitivity or radio-resistance, long-term cytotoxic test, such as clonogenic assay or long-term MTT assay, is the standard method [117]. In our present study, silencing of Notch1 did not change radiation sensitivity in four HNSCC cell lines examined by long-term MTT assay. One

possible reason is that the expression levels of Hes1 and Hey1 were compensated by co-expression of other *NOTCH* genes after knock down of Notch1 by siRNA transfection. Thus, Notch1 seems not to be causally involved in cisplatin- or radio-resistance in our HNSCC cell lines, although the expression of Notch1 is different between cisplatin resistant and sensitive cell lines.

4.6 Inhibition of Notch activation by γ-secretase inhibitors in HNSCC cells

The results from the evaluation of the effects of GSIs (GSI IX and GSI XII) on HNSCC cell viability raised another serious question. In our study, the growth of HNSCC cells could be significantly reduced by GSI XII, even in low concentrations. However, GSI XII did not markedly inhibit Notch1 cleavage. In contrast, GSI IX was much less toxic in the full range of tested concentrations. In a prior study [72], lymphoma cell viability was measured to investigate the effect of GSI XII on proliferation. Consistent with our findings, significant cell apoptosis was induced with very low concentration of GSI XII. GSI XII obviously induces cell death by downregulating NF-kB transcriptional activity. GSI IX had no detectable effect on cancer cell viability, compared with other GSIs [127]. Furthermore, from the results of the performed western blot, protein levels of C-Notch1 were obviously decreased in dose-dependent manners by GSI IX. Nearly 50% of C-Notch1 generation was blocked by GSI IX with a concentration of 30 μM. Actually, the range of inhibiting concentrations of GSIs vary among different GSIs and different target proteins. Generally, the effective inhibitory concentrations for Notch1 cleavage were always found to be higher than those concentrations shown for other proteins cleavages [128]. In a γ-secretase inhibitor assay, 0.1 μM of GSI could completely block Aβ generation, and only had very minor effect on Notch cleavage [128]. Also, the effect of inhibition depends on the type of GSIs and cancer cell type used in the study [129]. GSIs are altering the structure of γ-secretase and subsequently leading to potential changes in enzyme activity and specificity. These results indicated that GSIs differentially regulate the C-Notch1 in different tumor systems. With regard to the aim of this project, in which we wanted to block the Notch1 protein activity to investigate the role of Notch1 in cisplatin sensitivity in HNSCC cells, GSI IX was considered to be the better reagent for further investigation.

Notch1 pathway regulates the expression of various genes, few being oncogenic and few others being tumor suppressors [79]. In multiple myeloma cells, p21 expression was downregulated by GSIs and induced growth arrest providing enhanced sensitivity to chemotherapy reagents. However, in follicular lymphoma cells, when *HES1, c-Myc* and *cyclin D1* were found to be

downregulated with no observable inhibition of p21 after GSIs treatment, cancer cells were more sensitive to drugs. These results indicate that the biological effect of GSIs in different tumor cells depends on the target genes which are regulated by GSIs. We also examined expression levels of Hes1 and Hey1 expressions post GSI IX treatment. In contrast to the results of target gene expression after siRNA mediated knockdown of Notch1, Hes1 was suppressed in FaDu $_{CDDP-R}$ and UD-SCC-4 $_{CDDP-R}$ after the inhibition of C-Notch1. In addition, Hey1 was also suppressed in UD-SCC-4 $_{CDDP-R}$ but no inhibition of Hey1 was observed in FaDu $_{CDDP-R}$ cells. The ability of GSI IX to inhibit Hey1 might therefore render UD-SCC-4 $_{CDDP-R}$ cells more resistant to cisplatin. The ability of GSI IX to down regulate the Notch target genes might therefore result in different sensitivity rates compared to Notch1 siRNA transfection. The lack of down regulation of Hes1 and Hey1 might explain why specifically silencing of Notch1 did not change the sensitivity to either chemotherapy or radiation in HNSCC cells.

4.7 GSI IX decreases cisplatin sensitivity in HNSCC cells

We already confirmed that GSI IX inhibit the active NICD and its target genes. In the next step, we explored the effect of GSI IX on cisplatin sensitivity in HNSCC cells which potential as sensitizer for chemotherapy was shown in a previous study [112]. The authors investigated whether inhibition of Notch signaling pathway could affect the viability and sensitivity to chemotherapy in myeloma cells. GSI did not affect normal bone marrow cells at concentrations which are toxic for myeloma cells. GSI significantly improved the cytotoxicity of the chemotherapeutic drugs doxorubicin and melphalan. The sensitizer effect of GSI was mediated by *HES-1* and up-regulation of the pro-apoptotic protein Noxa. After this study, several studies investigated whether GSI could be used as a chemo-reagent sensitizer in cancer treatment. Most of these studies showed that GSIs significantly increased the sensitivity to chemotherapy, when combined with conventionally used chemotherapeutic agents (Summarized in Table 4.2). RO4929097 is an oral γ-secretase inhibitor used in clinical settings. In 2015, patients with refractory solid tumors received capecitabine with escalating doses of RO4929097 on a 21-day cycle in a multicenter phase I study [130]. Three patients with HNSCC were included in this study. Clinical benefit was observed in cervical and colon cancer, but not in these three HNSCC patients. However, the result did not show a detailed prognosis of each individual patient making it challenging to draw conclusion on the effect of this GSIs in HNSCC patients. It is a promising agent for cancer patient's management. But γ-secretase inhibitor is recommended to be used

carefully, especially for patients with HNSCC. For this, further experiments are needed to confirm the findings. Additional analysis *in vitro* show that GSIs themselves did not affect cell viability but resulted in enhanced taxane-induced G_2/M accumulation and apoptosis, leading to improve responses of human pancreatic ductal adenocarcinoma cells to taxanes [131]. Moreover, in HNSCC cells [132], GSI IX was shown to delay tumorigenesis and effectively reduce self-renewal of tumor stem cells. Further flow cytometric analysis showed a reduced cancer stem cell frequency by GSI IX mediated Notch1 inhibition, either alone or in combination with cisplatin, docetaxel, and 5-FU. However, not all the studies showed that GSIs could increase the chemotherapy sensitivity *in vitro*. Wang and colleagues presented that GSI IX decreased cisplatin-induced apoptosis by inhibition of Notch1 signaling pathway in osteosarcoma cells [133]. First part of their study, a total of eight osteosarcoma patients were enrolled and divided into two groups according to their cancer chemotherapeutic drug sensitivity. The result showed that osteosarcoma patients with higher Notch1 expression are more sensitive to cisplatin treatment indicating Notch1 functions as an oncogene in osteosarcoma cells. Furthermore, they used GSI IX to inhibit Notch1 to confirm their findings. Hes1 was suppressed approximately 50% in two osteosarcoma cell lines after the inhibition of Notch1. They found more surviving cells in the GSI IX treated group than the control group, indicating osteosarcoma cells become relatively resistant to cisplatin after the GSI IX treatment. Until now, this is the only study which showed similar results as my findings. Furthermore, inhibition by GSI IX in HNSCC cells even with cisplatin treatment might directly or indirectly decrease the expression and/or activity of caspase-3 protein, generally leading to an increased resistance to cisplatin. In consistent with my study, Wang et al found that the effect of GSI IX on tumor cells probably depends on the changes in the activity of caspase family, including caspase-3, caspase-8 and caspase-9 in these cells [133]. However, there were still some results differing between Wang et al' study and my study. When NICD was upregulated by NICD1 overexpression to activate the Notch1 signal pathway in osteosarcoma cells, the sensitivity to cisplatin was increased. They speculated that activation of Notch1 signaling pathway could lead to a higher cisplatin sensitivity in osteosarcoma cells. Due to the limited time for this thesis an overexpression of NICD1 in our HNSCC cell lines could not be performed. From their results, Wang and colleagues speculated that Notch1 might be the most important protein, which could be inhibited by GSIs in osteosarcoma cells. However, in present study, the sensitivity to cisplatin was not changed after Notch1 knockdown by siRNA but by GSI IX treatment which raised the question if other proteins instead of Notch1 are involved in this biological phenomenon.

Table 4.2 GSI IX and sensitivity to chemotherapy reagents in cancer cells *in vitro*

Refs	Cancer cell lines	GSIs	Effect on sensitivity	Chemotherapy reagents	Implication
[112]	Myeloma	GSI XII	Increase	doxorubicin and melphalan.	Apoptosis
[120]	Breast cancer	GSI IX	Increase	Paclitaxel	N.R
[131]	Pancreatic ductal adenocarcinoma	GSI IX	Increase	Taxanes	Cell cycle and apoptosis
[132]	HNSCC	GSI IX	Increase	Cisplatin, docetaxel and 5-FU	Stemness
[133]	Osteosarcoma	GSI IX	Decrease	Cisplatin	Apoptosis
[134]	Lung adenocarcinomas harboring EGFR mutations	GSI	Increase	Gefitinib	Apoptosis
[135]	Triple negative breast cancer.	GSI	Increase	Doxorubicin	Apoptosis
[136]	Breast cancer	GSI IX	Increase	Doxorubicin	Apoptosis

4.8 GSI IX slightly decreases radio-sensitivity in HNSCC cells

The radio-sensitivity was only slightly decreased by GSI IX treatment in four HNSCC cell lines, whereas GSI IX could largely decrease cell sensitivity to cisplatin. Theoretically, factors that render a tumor cell resistant to one drug may also simultaneously increase resistance to several other treatments, such as irradiation. As mentioned before, although both cisplatin and irradiation caused cell death by targeting DNA, the mechanism of cisplatin resistance and irradiation resistance is different [48, 137]. In preclinical studies in cancer cell lines and xenografts, cross-resistance between chemotherapy and radiotherapy was frequently seen, but this was not an universal observation [138-141]. Maybe that is the reason why GSI IX did not interfere to the same extent with cisplatin and radio-sensitivity in this study.

Furthermore, our observation is in disagreement with two earlier studies which investigated the role of GSIs in radio-resistance. Yu and colleagues found that GSI IX could significantly inhibit nasopharyngeal carcinoma (NPC) cell growth and improve radio-sensitization [142]. Another study also found that treatment with GSI I after radiation can significantly enhance radiation-mediated lung cancer cytotoxicity [143]. The MTT assay performed in these two studies was short-term assay. Furthermore, cancer cells were treated with radiation 24 hours before GSI administration in the second study. In my study, cancer cells were treated with radiation 24 hours after GSI administration. Maybe that is another reason to explain the difference among these

studies. It remains to be investigated if the inhibition of Notch1 differently affects radio-sensitivity if given before or after irradiation.

4.9 Notch2-4 expression in HNSCC cells

Since knockdown of Notch1 did not show similar effect as GSI IX on cisplatin sensitivity, we hypothesized that another Notch protein might be involved in cisplatin chemo-resistance. Therefore, mRNA levels of Notch2, Notch3 and Notch4 were examined in HNSCC cell lines. Compared to Notch1, there was more Notch3 expressed in HNSCC cells. Furthermore, the most prominent differences between the sensitive and resistant cell lines were detected for Notch3. In contrast, the expression of Notch4 was very low. Generally, the role of Notch2 and Notch4 were rarely investigated in HNSCC. We decided to examine the role of Notch3 in cisplatin sensitivity in the next step.

In many of epithelial malignancies such as pancreas, ovarian and lung cancers, Notch3 is considered to play a key role in cell differentiation [144-146]. In tongue cancer cells [86], normal tongue epithelial cells were completely negative for Notch3 protein expression, whereas positive immunostaining was observed for Notch3 in the cancer cells. However, there was no significant difference in Notch3 expression between patients with or without cervical lymph node metastasis. In my study, similar expression pattern was observed for Notch3 between FaDu and FaDu $_{CDDP-R}$ cells. The UD-SCC-4 $_{CDDP-R}$ expressed higher levels of Notch3, compared with UD-SCC-4, indicating Notch3 might be involved in cisplatin resistance in UD-SCC-4 cells.

4.10 Silencing of Notch3 did not change the sensitivity to cisplatin in HNSCC

The data of this study provided evidence that inhibition of Notch3 could not change cisplatin sensitivity in HNSCC cells. One recent study investigated the role of Notch3 in cisplatin sensitivity in EBV-associated nasopharyngeal carcinoma cells (NPC) [147]. They found that Notch3 was higher expressed in all EBV-positive NPC cell lines and most of primary tumors, compared to other Notch receptors. The ability of NPC cells to form spheroids was also significantly decreased after knockdown of Notch3 expression. Notably, Notch3 knockdown highly enhanced the sensitivity of NPC cells to cisplatin treatment. The MTT assay in this study was performed as short-term assay. From my experience, with the applied low seeding tumor cell number, most cells cannot tolerate the treatment. Nevertheless, the lacking down regulation of Hes1 and Hey1 in this study might explain why silencing of Notch3 did not change the sensitivity to cisplatin chemotherapy in HNSCC cells. Further experiments have to be performed to understand the Notch signaling pathway and its role in resistance mechanisms.

4.11 Conclusions

The study could demonstrate that HNSCC cells express F-Notch1 and active Notch1 protein. Notch1 protein might play a critical role in acquired resistance to cisplatin in HNSCC cells. Long-term treatment of HNSCC cells with low concentration of cisplatin only induced cisplatin resistance, but did not increase the cross-resistance to radiation in these cells. Inhibition of Notch1 did not alter cisplatin or radio-sensitivity in HNSCC cells. Furthermore, inhibition of Notch3 also did not alter cisplatin or radio-sensitivity in HNSCC cells. Further experiments are needed to investigate the role of Notch signaling in HNSCC cells and the cisplatin resistance mechanisms in this tumor entity.

Additionally, silencing of Notch1 and Notch3 by siRNA transfection did not change the sensitivity to cisplatin in HNSCC cells. When cells treated with GSI IX before cisplatin, its sensitivity was significantly deceased. This is important to further check the role of GSIs in HNSCC as GSI IX has already been used in HNSCC patients in phase I clinical trials as chemotherapy sensitizer.

In conclusion, elucidating the function of Notch signaling pathway in HNSCC may will enable the development of promising therapies and maybe also stratification of patients from mutational status or Notch1 expression for a better choice of therapy and improvement of clinical diagnostics.

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Affidavit

"I, Sheng liming, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic 'The role of Notch signalling pathway in cisplatin sensitivity and radiation sensitivity in head and neck squamous cell carcinoma' 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 interest 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 Signature

Declaration of any eventual publications

[Name of the doctoral candidates] had the following share in the following publications:

Publication 1: [Authors], [titles], [magazine], [year of publication]
Contribution in detail (please briefly explain):

Publication 2: [Authors], [titles], [magazine], [year of publication]

Contribution in detail (please briefly explain):

Publication 3: [Authors], [titles], [magazine], [year of publication]
Contribution in detail (please briefly explain):

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

_____

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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