S100 Gene Family Members in Oral Squamous Cell Carcinomas (OSCCs):

Functional Characterization of S100A14 in Proliferation and Invasion of OSCC Derived Cells

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Dissertation for the degree of Philosophiae Doctor (PhD)

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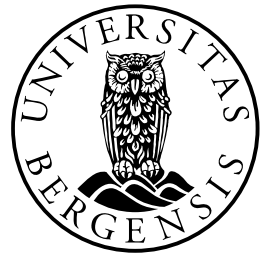

Dissertation for the degree of Philosophiae Doctor (PhD)

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'This work is dedicated to my parents,

brother and sisters'

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Bergen, September, 2010

Dipak Sapkota

Summary

It is generally accepted that carcinogenic substances in the form of tobacco, alcohol or oncogenic viruses, *etc* cause genetic changes resulting in conversion of normal oral epithelium to a potentially malignant (dysplastic) lesion, and subsequently into the invasive oral squamous cell carcinoma (OSCC). However, the precise molecular mechanism underlying OSCC carcinogenesis remains unclear. Several members of the multifunctional Ca^{2+} binding S100 proteins have been described in connection with a range of human cancers, including OSCCs. Studies from our group, using high throughput genomic and proteomic methods, have previously identified differential expression of members of the S100 proteins in OSCCs from different populations. S100A14 is a recently identified member of the S100 protein family. Although differential expression of S100A14 has been described in different human cancers, its biological roles in carcinogenesis have not been well characterized. This study aimed (i) to examine the mRNA expression profile of 16 of the *S100* gene family members in OSCCs and (ii) to characterize the possible role(s) of S100A14 in proliferation and invasion of OSCC derived cells.

We identified significant down-regulation of *S100A4*, *S100A6*, *S100A8* and *S100A14* mRNAs in OSCCs compared to their pair-wised controls. Down-regulation of S100A14 was further validated at the protein level in OSCC archival tissues using immunohistochemistry, and in an *in vitro* oral cancer progression model both at the mRNA and protein levels. To investigate the functional roles of S100A14, we employed retroviral vector mediated over-expression and siRNA mediated knock-down of the endogenous S100A14 in two OSCC derived cell-lines (CaLH3 and H357). S100A14 over-expression resulted in a significant reduction in CaLH3 cell proliferation due to G1-phase cell cycle arrest, but not apoptosis. This G1- arrest was found to be associated with nuclear accumulation of the tumor suppressor protein p53 and p53-dependent up-regulation of p21. These findings suggest a functional link

between S100A14 and cell-cycle regulators p53 and p21 in the regulation of cell cycle in OSCC derived cells and support the idea that S100A14 might function as a tumor suppressor protein working in the p53 pathway.

Characterization of the role of S100A14 in tumor invasion showed that over-expression of S100A14 resulted in a significant decrease in the invasive potential of the OSCC derived CaLH3 and H357 cell-lines, whereas siRNA mediated knock-down resulted in a significant increase in the invasive potential of the CaLH3 cell-line *in vitro*. PCR array and validation using qRT-PCR and gelatin zymography revealed that S100A14 over-expression was associated with down-regulation of *MMP1* and *MMP9* mRNAs in both CaLH3 and H357 cell-lines and suppression of MMP9 activity in the CaLH3 cell-line. Additionally, an inverse correlation between mRNA expression levels of *MMP1* and *MMP9* with *S100A14* was found in OSCC tissue samples. These findings suggest that S100A14 negatively regulates expression and activity of MMP1 and MMP9 and that might be responsible for the S100A14 mediated regulation of tumor cell invasion. In conclusion, findings of this work suggest that differential expression of several of the *S100* gene family members is a common genetic alteration in OSCCs. S100A14, similar to other members of the S100 family, is involved in key cellular functions such as cell cycle regulation and tumor cell invasion indicating a tumor suppressor role for S100A14. Uncontrolled cell proliferation and invasion, characteristics of OSCCs, might therefore be related to altered expression of S100A14 frequently observed in these cancers.

List of abbreviations

CAM	Cell adhesion molecules
CDK	Cyclin dependent kinase
CDKi	Cyclin dependent kinase inhibitor
ECM	Extracellular matrix
FDR	False discovery rate
HNSCC	Head and neck squamous cell carcinoma
IHC	Immunohistochemistry
MMP	Matrix metalloproteinase
NHOM	Normal human oral mucosa
ODL	Oral dysplastic lesion
OSCC	Oral squamous cell carcinoma
qRT-PCR	quantitative Reverse transcription-polymerase chain reaction
SAM	Significance analysis of microarray
shRNA	short hairpin Ribonucleic acid
siRNA	small interfering Ribonucleic acid
sRT-PCR	semiquantitative Reverse transcription-polymerase chain reaction
TIMP	Tissue inhibitors of matrix metalloproteinase
TP53	Tumor protein 53
wt	wild type

List of publications

This thesis is based on the following original publications referred to in the text by their roman numerals

Paper I
Sapkota D, Bruland O, Bøe OE, Bakeer H, Elgindi OAA, Vasstrand EN, Ibrahim SO (2008). Expression profile of the S100 gene family members in oral squamous cell carcinomas. *J Oral Pathol Med* **37:** 607-615.

Paper II
Sapkota D, Costea DE, Blø M, Bruland O, Lorens JB, Vasstrand EN, Ibrahim SO. S100A14 induces G1-arrest through nuclear accumulation of p53 and p53 –dependent up-regulation of p21 in oral carcinoma derived cells *(manuscript).*

Paper III
Sapkota D, Bruland O, Costea DE, Haugen H, Vasstrand EN, Ibrahim SO. S100A14 regulates the invasive potential of oral squamous cell carcinoma derived cell-lines *in vitro* by modulating expression of matrix metalloproteinases, MMP1 and MMP9 *(Eur J Cancer, In Press)*.

1. Introduction

1.1. Oral squamous cell carcinoma (OSCC)

Head and neck cancers encompass malignancies that arise in the oral cavity, nasal cavity, para nasal sinuses, pharynx and larynx. Oral cancers represent 40% of all head and neck cancers and consist of the malignancies arising in the lip, tongue, floor of the mouth, gingiva, palate and buccal mucosa [1, 2]. The most frequent neoplasms arising from the oral epithelium are oral squamous cell carcinomas (OSCCs), representing more than 90% of all oral cancers. Combined with pharyngeal cancers, oral cancers rank as the sixth most common type of cancer world-wide, being the third most common in the developing countries (for example, South and Southeast Asian countries: Pakistan, India, Sri Lanka, Taiwan; African countries: Sudan, *etc*) [3]. This wide geographical variation in the incidence of OSCCs has been linked with the country specific risk factors, for example: betel quid and smokeless tobacco in South and Southeast Asian countries [4] and *toombak* in the Sudan [5, 6]. Several etiological factors namely use of tobacco (smoked and smokeless) and alcohol, infection with human papilloma virus (HPV) or herpes simplex virus (HSV), dietary deficiencies or imbalances (micronutrient deficiency), genetic predisposition, *etc* have been linked with the development of OSCCs. Based on the available global evidence, Warnakulasurya S. has categorized risk factors for OSCCs into *established*, *strongly suggestive*, *possible* and *speculative factors* (**Table 1**) [7].

Table 1: Suggested risk factors for OSCCs (adapted from Warnakulasurya S [7])

Established	Strongly Suggestive	Possible	Speculative
Smoking	Sunlight (lip)	Viruses	Mouthwashes
Tobacco chewing	Radiation	Immune deficiency	Periodontal diseases
Snuff (*Toombak*) dipping		Dentition?	Mate drinking
Alcohol misuse		Ethnicity?	
Betel quid use			

1.2. Potentially malignant oral disorders

Oral carcinogenesis is believed to evolve as a multi-step process where the majority of OSCCs are thought to be preceded by or associated with potentially malignant (dysplastic) oral epithelial lesions. Several terms 'pre-cancer', 'precursor lesions', 'intra-epithelial neoplasia' and 'potentially malignant' have been used broadly and interchangeably to describe the clinical presentation of oral lesions that may have the potential to transform into cancer. Recently, the term 'potentially malignant disorders' has been recommended when referring to these lesions [8]. Leukoplakia (white patches) and erythoplakia (red patches), the most common form of potentially malignant oral disorders, carry high risk of malignant transformation. Malignant transformation rates up to 36% and 50% have been reported respectively for leukoplakia and erythoplakia [reviewed in 9]. Malignant transformation rates of these lesions are often correlated with the severity of the histological (dysplastic) changes. In addition, several molecular alterations have been identified and correlated with the malignant potential of these lesions. Despite the progress in the field of molecular biology, no single or a set of molecular markers can reliably predict the malignant transformation rates of oral premalignant lesions [10].

1.3. Molecular biology of OSCC

Genetic damage lies central to the carcinogenesis process. It is generally accepted that alterations (usually mutations) in three classes of genes namely, oncogenes, tumor-suppressor genes and stability genes are responsible for the development of human cancers [reviewed in 11]. Oncogenes are the genes whose protein products are either produced in higher amounts or have higher activity, hence acting in a dominant manner (gain of function). Oncogenes can be activated by chromosomal translocations or insertional mutagenesis, gene amplifications or by activating point mutations/deletions. On the other hand, tumor suppressor genes are the genes whose protein products have loss of function due to mutations and are recessive in

nature. Missense mutations at residues that are essential for the activity of protein, deletions or insertional mutations or epigenetic silencing result in loss of function of tumor suppressor genes. The stability genes or caretakers are involved in the repair of genetic damage induced during normal cell division or due to exposure with carcinogenic environment. Stability genes are also inactivated in a manner similar to that of the tumor suppressor genes [11, 12]. Mutations in these classes of genes ultimately result in the development of an invasive cancer characterized by self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, invasion and metastasis to local and distant organs, limitless replicative potential and sustained angiogenesis [13].

The oral cavity is frequently exposed to high levels of carcinogenic substances in the form of tobacco (such as benzo-(a)-pyrene and nitrosamines), alcohol or infection with oncogenic viruses, such as HPV. Consequently, and similar to other cancers, OSCCs also develop as a result of genetic changes caused by these carcinogens. It is generally accepted that combinations of these genetic changes initially result in the development of potentially malignant (dysplastic) oral lesion which may progress to invasive carcinoma under sustained additional genetic alterations [14]. Based on the genetic alterations reported in potentially malignant (dysplastic) oral lesions and oral cancers, Califano and colleagues have proposed a genetic progression model for head and neck squamous cell carcinoma (HNSCC) [14][reviewed in 15] (**Figure 1**). According to the model, a loss of chromosomal region 9p ($p16^{INK4a}$ and $p14^{ARF}$) has been described in 70-80% of oral dysplastic lesions, and together with the inactivation of the remaining alleles of $p16^{INK4a}$ and $p14^{ARF}$ by promoter hypermethylation, it represents one of the earliest and the most frequent events involved in OSCC progression [14, reviewed in 15 and 16]. Loss of heterozygosity (LOH) of *TP53* has been reported as early as in the dysplastic stage of oral carcinogenesis [14].

Recently, we conducted multi-country studies using high throughput molecular techniques like microarray, comparative genomic hybridization (CGH) and proteomics to dissect the possible changes in global gene and protein expression profiles, chromosomal abnormalities and deregulated molecular networks occurring during the transition from normal oral epithelium to OSCC. Alterations in the chromosomal regions harboring several members of the *S100* and Matrix metalloproteinase *(MMP)* gene families were found to be common in the HNSCC/OSCC samples examined [17] (Suhr *et al*; unpublished data). Accordingly, differential mRNA and protein expression levels of members of the *S100* and *MMP* genes were found in the HNSCCs/OSCCs examined [18-20]. We have also reported prevalence of HPV, HSV, and Epstein-Barr virus (EBV) DNA in a large number of potentially malignant (dysplastic) oral lesions/cancers examined from different populations suggesting their role in OSCC carcinogenesis [21, 22]. A predominance of differential expression of several members of the S100 proteins in the samples examined attracted our attention in understanding their possible role in OSCC carcinogenesis.

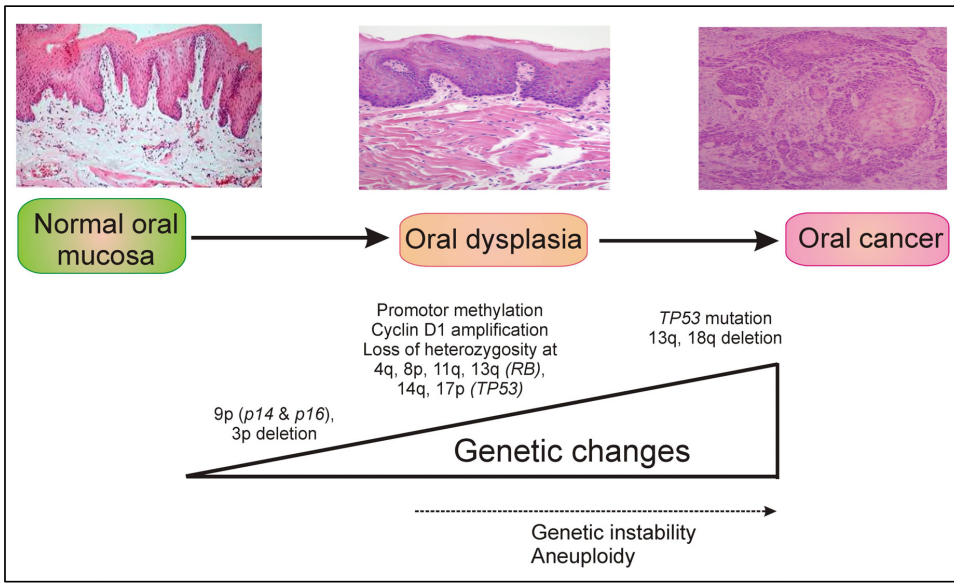

Figure 1. Proposed multi-step genetic progression model of oral carcinogenesis (modified from Califano J *et al*; 1996 [14] and Choi S *et al*; 2007 [15]).

1.4. S100 gene family members

Moore identified for the first time an unfractionated mixture of the S100B and S100A1 from the bovine brain and called it 'S100' because the mixture was soluble in 100% saturated solution of the ammonium sulphate solution [23]. S100 proteins are small acidic proteins (10-12 kDa) that constitute the largest subfamily of calcium binding proteins of the EF-hand type. Among the 25 members (with a sequence homology of 25-65%) described till now, 21 members (A100A1-S100A18, trichohylin, filaggrin and repetin) are clustered at chromosome locus 1q21, while the remaining members are located at loci 4p16 (S100P), 5q14 (S100Z), 21q22 (S100B) and Xp22 (S100G) [24, reviewed in 25, 26].

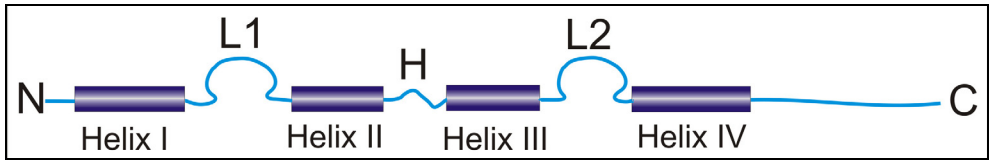

Figure 2. Schematic illustration of the typical structure of the S100 protein (L1 and L2: calcium binding loops, H: hinge region, N and C: N- and C-terminals) (modified from Donato R, 2001 [27])

Structural analyses have revealed that most of the S100 proteins exist in cells as anti-parallel homo/hetero dimers. The S100 protein monomer consists of two calcium (Ca^{2+}) binding motifs of the EF-hand type separated by a flexible region, the hinge (**Figure 2**). Each Ca^{2+} binding motif consists of a Ca^{2+} binding loop flanked by two α-helices. Helices I and II flank the Ca^{2+} binding loop in the N-terminal site whereas the Ca^{2+} binding loop in the C-terminal site is flanked by III and IV helices. IV helix in the C-terminal site is followed by a C-terminal extension. The C-terminal extension and the hinge region have the most sequence variability among the S100 proteins. Upon Ca^{2+} binding, the S100 dimer undergoes conformational change due to the reorientation of helices III and IV and forms clefts like target protein binding sites [28, 29]. Interacting with their target molecules in a Ca^{2+} -dependent as well as an independent manner, S100 proteins regulate a range of biological

activities such as cell growth, cell motility, signal transduction, transcription, apoptosis and cell survival (**Table 2**).

Table 2. Suggested functions and disease associations of selected members of the S100 protein family

S100 protein	Postulated functions	Disease association [Ref.]
S100A1	Regulation of cell motility, muscle contraction, Phosphorylation, neurite overgrowth, regulation of Ca^{2+}	Cardiomyopathies [30]
S100A2	Tumor suppressor functions, interaction with p53	Cancer [30, 31]
S100A3	Hair shaft formation, inverse association with tumor differentiation and TNM stage	Hair damage, Cancer [30, 32]
S100A4	Regulation of cell invasion, angiogenesis, cell proliferation, apoptosis, interaction with p53	Cancer [30, 33, 34]
S100A5	Ca^{2+}, Zn^{2+} and Cu^{2+}- binding protein, association with tumor recurrence	Cancer? [30, 35]
S100A6	Regulation of cell growth and apoptosis, regulation of cytoskeletal dynamics, interaction with p53	Cancer [30, 36, 37]
S100A7	Regulation of keratinocyte proliferation and maturation, inflammation	Psoriasis, Cancer [30]
S100A8/A9	Chemotactic activities, myeloid cell differentiation and maturation, arachidonic acid metabolism	Inflammatory disorders, Cancer [30]
S100A10	Regulation of membrane traffic: ion channels, regulation of phospholipase A2	Depressive disorders, Inflammation [38, 39]
S100A11	Regulation of kerationcyte proliferation, role in keratinocyte cornified envelope formation, regulation of cytoskeletal components	Cancer, Inflammation [30, 40]
S100A12	Interaction with RAGE-inflammatory response, chemotactic functions	Inflammatory conditions, Autoimmune disease [30, 41]
S100A13	Regulation of FGF-1 release, association with tumor cell invasion	Tumor angiogenesis, Invasion [30, 42]
S100A14	Association with tumor invasion, differentiation	Cancer? [43]
S100B	Cell motility, cell proliferation, survival and differentiation, regulation of Ca^{2+}, extracellular functions, such as: neurite extension, interaction with p53	Developmental brain dysfunction, Alzheimer's Disease, Down's syndrome, Depression, Cancer [30, 44]

A number of molecules including enzymes, cytoskeletal components, and transcription factors have been identified as targets for the S100 proteins [25, 27, 30]. The following observations (i) occurrence of frequent structural and numerical aberrations in the chromosomal region 1q21 [45] (where most of the *S100* gene members are located [24]) in human cancers, (ii) altered mRNA and protein expression levels of several of the S100 members in different human malignancies [46] and (iii) involvement of the S100 proteins in several biological functions (cell growth, cell motility, signal transduction, transcription, apoptosis and cell survival) related to normal development and tumorigenesis [27, 47, 48]; suggest that these proteins are closely related to human malignancies. Accordingly, in previous work from our group chromosomal rearrangements have been identified in the 1q12 chromosome locus and differential expression of S100A1 and S100A2 members in HNSCCs/OSCCs from different populations has been reported [17-19] (Suhr *et al*; unpublished data). Further comprehensive expression profiling of 16 of the *S100* gene members carried out in this study identified *S100A14*, a recently discovered *S100* gene member, to be frequently down-regulated in OSCCs and in the OSCC derived cell-lines.

1.4.1. S100A14

S100A14 was first identified in 2002 by analyzing human lung cancer cell lines [26] and subsequently in 2003 as a membrane-associated protein in breast cancer cells [49] (hence it is also known as Breast Cancer Membrane Protein 84, BCMP84). The *S100A14* gene has been mapped to human chromosome 1q21 and reported to contain 4 exons and 3 introns encoding for a small acidic EF-hand type Ca^{++} binding protein (104 amino acids) with a predicted molecular weight of 11.66 kDa [26]. S100A14 shares significant similarities as well as differences with other S100 members. S100A14 shares 68% similarity and 38% identity to S100A13 and with other S100 members like S100A4 (62% similarity; 30% identity), S100A2 (60% similarity; 30% identity), S100A10 (58% similarity; 31% identity), and S100A9 (55%

similarity; 34% identity) [26]. However, in contrast to other S100 members which consist of 3 exons and 2 introns (except *S100A5*, consisting of 4 exons with exon 3 and 4 being the coding ones; *S100A4*, containing an additional alternatively spliced untranslated exon; and *S100A11*, with the coding sequence beginning already in the first exon), *S100A14* contains 4 exons and 3 introns with exons 2-4 being the translated ones [26]. Similarly, the Ca^{2+} binding loop at the N-terminal of the S100A14 protein contains 13-amino-acids loop which is in contrast to the 14-amino-acids loop characteristic of the S100 protein family [26]. Moreover, the Ca^{2+} binding loop at the C-terminal has been reported to carry mutations thus handicapping the Ca^{2+} binding ability of the S100A14 [26].

Sequences upstream of the transcription initiation site of the *S100A14* have been shown to contain consensus recognition sequences for a number of transcriptional factors like c-Myc/Max, AREB6, USF and E2 box repressor deltaEF1 [26]. Recently, p53 has been identified as a transcriptional regulator of *S100A14* [50]. In addition, the S100A14 protein has been predicted to contain a number of post-translational modification sites for example: N-glycosylation, protein kinase phosphorylation, casein kinase II phosphorylation and N-myristoylation [26, 51]. These observations indicate that S100A14 is under tight transcription and post-translational control and these controlling mechanisms might be important for the regulation of S100A14 expression and function. Moreover, differential expression of S100A14 found in different human normal and tumor tissues [26, 43, 52, 53] suggests that its regulation may be tissue and context specific and its expression might be de-regulated in pathological conditions including human cancers. Nevertheless, the biological functions and molecular targets of S100A14 are largely unknown.

1.4.2. Expression profile and sub-cellular localization of S100A14

A variable abundance in the expression of the *S100A14* transcript has been shown in several types of normal human and cancer tissues and cell-lines. Normal human colon tissue was

shown to express the highest amount of *S100A14* mRNA, whereas moderate expression levels were reported in normal human tissues such as thymus, kidney, liver, small intestine, lung, breast, ovary, prostate, rectum, stomach, thyroid and uterus. In contrast, low *S100A14* mRNA expression has been reported in normal heart and no expression in normal human brain, skeletal muscle, spleen, placenta and peripheral blood leukocytes [26]. Ovary, breast, uterus, lung, prostate and thyroid tumors have been shown to over-express *S100A14* mRNA [26]. On the other hand, down-regulated expression of *S100A14* mRNA has been demonstrated in kidney, rectum, colon and stomach tumors, oesophageal carcinoma, colorectal carcinoma and OSCCs [26, 43, 52, 53]. Cytoplasmic and perinuclear localization of S100A14 protein has been shown in cells derived from lung tumors [26]. Conversely, strong and weak membranous staining has been reported in normal and colorectal carcinoma tissues respectively [43]. Further, membranous to cytoplasmic translocation of S100A14 protein from the highly differentiated to the poorly differentiated (invading islands of cells) areas has been described in squamous cell tonsil carcinoma and bladder papillary transitional cell carcinoma [49].

1.4.3. Biological functions of S100A14

The biological functions of the S100A14 are largely unknown. Nevertheless, the following observations suggest that S100A14 might be important in multiple biological functions: (i) S100 family members are 'highly conserved multifunctional' proteins [47] and S100A14 is a member of this family, (ii) S100A14 is found to be differentially expressed in different tissue types and human cancers, (iii) *S100A14* is transcriptionally regulated by the tumor suppressor protein p53 [50], (iv) S100A14 has been shown to be localized in the plasma membrane, cytoplasm and perinuclear area with sub-cellular translocation in some of the tumor types. In support of this suggestion, decreased immunoexpression of S100A14 has been correlated with poor differentiation and high metastatic potential of colorectal carcinomas [43].

1.5. Cell cycle regulation

The sequence of stages through which a living cell passes between one cell division and the next is called the cell cycle. This consists of two main phases: Interphase and M-phase. The interphase further consists of G0, G1, S and G2-phases where as M-phase includes mitosis and cytokinesis. G0 is the quiescent phase where the living cell no longer divides [12]. Cyclins and cyclin dependent kinases (CDKs) are the key players of cell cycle regulation. Cyclins form complexes with CDKs and the resulting Cyclin/CDK complexes are necessary for the cell to pass through the specific phases of cell cycle. However, normal cells tightly regulate cell cycle progression by incorporating specific check points (G1, G2 and M-checkpoints) which sense signaling cues and respond either by allowing cell cycle to progress or by halting it until the errors are fixed, or alternatively by inducing the onset of apoptosis. CDK inhibitors (CDKi) [CIP/KIP family: $p21^{WAF1/CIP1}$ (p21), $p27^{KIP1}$ (p27), $p57^{KIP2}$ (p57) and INK family: $p15^{INK4b}$ (p15), $p16^{INK4a}$ (p16), $p18^{INK4c}$ (p18), $p19^{INK4d}$ (p19)] provide one of the most important mechanisms for inactivating CDKs and thereby blocking the cell cycle at specific checkpoints. Among these inhibitors, p21, p27 and p16 are considered to be important for regulation of the G1 check point [54-56] (**Figure 3**).

1.5.1. p53, p21 and p27 proteins

The tumor suppressor protein p53

Cells are regularly challenged by various cellular stress conditions such as radiation-, drug-, or carcinogen-induced DNA damage or oncogene activation (Ras, Myc) or other cellular stresses like hypoxia and nucleotide depletion. These conditions may nurture tumor initiation. p53, the guardian of the genome, responds to these insults by eliciting a number of key cellular processes (cell cycle arrest, apoptosis, DNA repair, inhibition of angiogenesis), thereby protecting the cells from malignant transformation [reviewed in 57 and 58] (**Figure 3**). One of the major effects of p53 activation by upstream pathways is the induction of cell cycle arrest. p53 directly stimulates expression of *CDKN1A* (p21) and brings about the G1-phase cell cycle arrest (**Figure 3**) [59]. p53 also induces G2-phase cell cycle arrest by activating transcription of *Gadd45, 14-3-3σ* and along with *CDKN1A* [57].

Under normal conditions, the amount of p53 protein in cells is kept at a low level, primarily due to ubiquitin proteasome mediated degradation [57, 60]. Once the cells are challenged by stress conditions, p53 escapes the degradation pathway and undergoes stabilization and nuclear accumulation. Regulation of p53 stabilization is one of the most effective mechanisms of controlling p53 activity. Stabilization of the p53 is a complex process which largely depends on the p53 negative regulator, Mdm2 (an E3-ubiquitin ligase). In addition to Mdm2, stability of p53 appears to be regulated by activities of other E3 ligases (pirh2 and Cop-1), deubiquitinases (HAUSP), regulatory proteins (MdmX, ARF), ubiquitin analogues (SUMO1, Nedd8) and post-translational modifications of p53 [57, 60]. Recently, several members of the S100 protein family (such as S100A2, S100A6, S100B) have been shown to interact with the p53 and these interactions have been suggested to be important for p53 stabilization, its nuclear accumulation and activity [37, 61, 62].

Tumor cells, however, acquire certain mechanisms to inactivate the activity and functions of the p53. In more than 50% of human cancers, p53 is directly inactivated by mutations of the *TP53* gene [63]. In other cases, p53 is indirectly inactivated either by binding to viral proteins or by alterations in the genes whose protein products interact with p53 or convey information to or from p53 or by mislocalization of p53 to cytoplasm [reviewed in 57].

p21 protein

The CDKi p21 is encoded by the *CDKN1A* gene. One of the important functions of p21 is the control of cell proliferation by regulating cell cycle kinetics. It specifically blocks the kinase activity of CDK2 and prevents the Cyclin E/CDK2 complex from phoshorylating Rb protein and thus inhibiting dissociation of E2F from Rb and E2F dependent transcription of genes necessary for DNA replication [64, 65] (**Figure 3**). Moreover, by binding with PCNA, p21 also inhibits DNA synthesis [66].

Although mutations of the *CDKN1A* gene are rare events [67, 68], altered expression of p21 is a frequent finding in human malignancies including OSCCs [69, reviewed in 70 and 71]. Expression of p21 is regulated both at the transcriptional and post-transcriptional levels [reviewed in 71]. p53 is one of the main transcriptional activators of p21. In response to cellular stresses, p53 activates transcription of *CDKN1A* and subsequently brings about the G1-phase cell cycle arrest [59]. Apart from p53-dependent regulation, several p53-independent pathways also regulate transcription of p21 [reviewed in 71]. p21 is also subjected to post-transcriptional modification by proteasome dependent degradation and phosphorylation with subsequent cytoplasmic mislocalization [reviewed in 71].

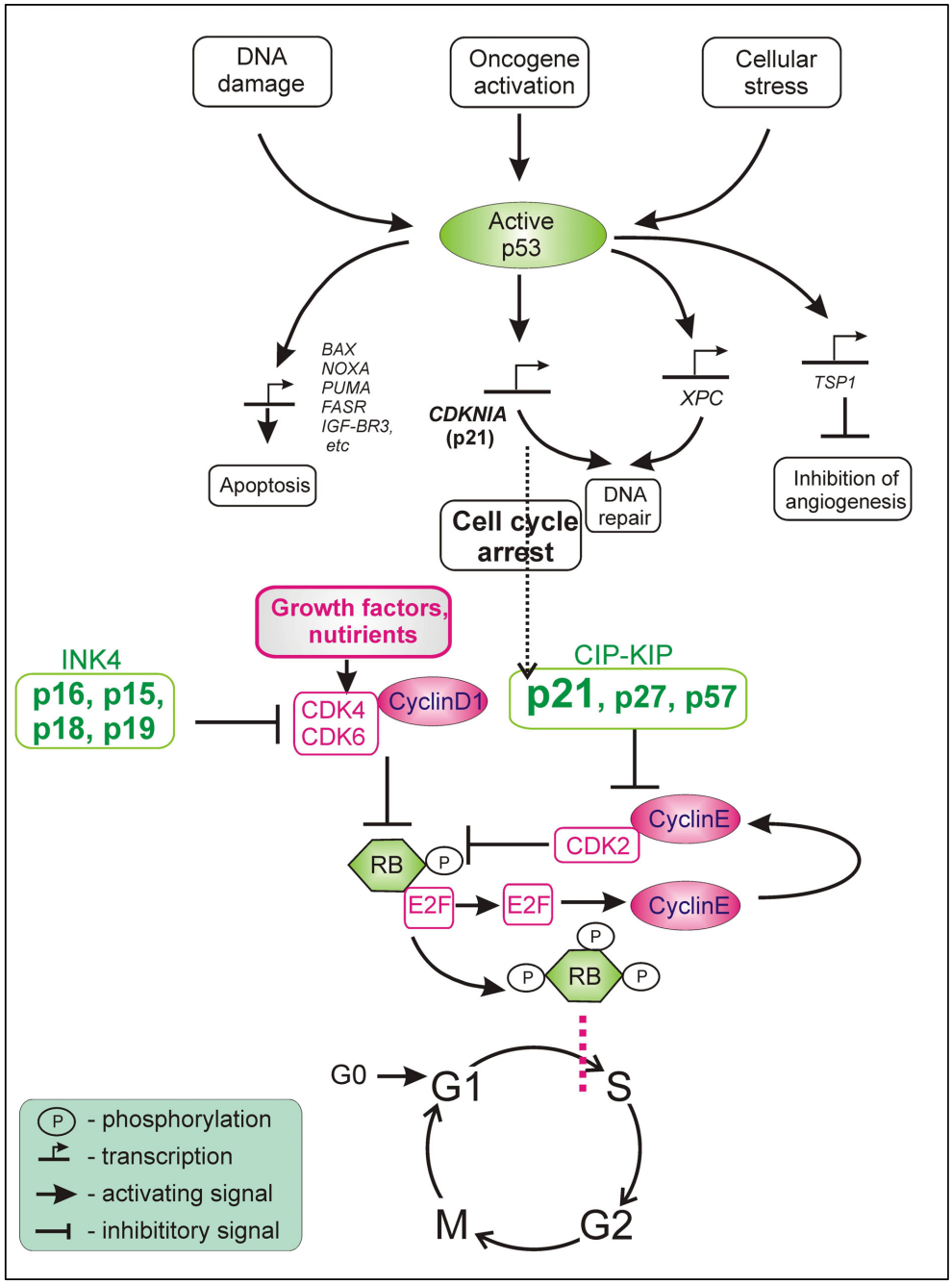

Figure 3. Simplified presentation of up- and down-stream signaling pathways of p53 and its interconnection with G1-phase cell cycle control through p21. Negative and positive regulators of cell cycle are marked respectively with green and red letters and/or boxes (modified from Pecorino L, 2006 [12] and Lapenna S *et al*; 2009 [72]).

p27 protein

p27, encoded by the *CDKN1B* gene, was initially identified as an inhibitor of the cyclin E-CDK2 complex [73]. Similar to p21, p27 blocks the activity of the Cyclin E/CDK2 complex resulting in G1-phase cell cycle arrest [73, 74] in response to growth factor deprivation, contact inhibition and loss of adhesion to extracellular matrix (ECM) (**Figure 3**) [75].

Similar to p21, somatic mutations of the *CDKN1B* gene are rare events in human malignancies [76]. However, down-regulated expression of p27 is commonly found in human malignancies and is often correlated with poor clinical outcomes [77-79]. Regulation of p27 activity is complex and is determined by the intracellular expression of p27, its distribution among different cyclin-CDK complexes and its sub-cellular localization. Although p27 expression is regulated both at the transcriptional [80, 81] and translational levels [82, 83], ubiquitin dependent proteolysis is thought to be the primary mechanism determining the cellular p27 protein level [84].

1.5.2. S100 proteins, cell cycle regulation and tumor growth

Differential expression of several members of the S100 protein family is a frequent finding in human malignancies. Several of the S100 protein members (S100A2 [85], S100A4 [reviewed in 86], S100A6 [87], S100A7 [reviewed in 88], S100A8/A9 [89], S100A11 [90] [reviewed in 40]) have been linked with tumor proliferation and growth. Uncontrolled tumor growth, one of the hallmarks of human cancer, is frequently associated with impaired cell cycle control [13]. Although several of the S100 protein family members have been linked with regulation of the cell cycle control, the precise molecular mechanisms involved are, however, not fully understood. S100A2 is found to be frequently down-regulated in cancers and is considered as a putative tumor suppressor protein [46]. Forced over-expression of S100A2 has been shown to inhibit cellular proliferation associated with G1- and S-phase cell cycle arrest in KB cells [85]. Similarly, S100A4 knock-down mediated suppression of pancreatic cancer cell growth

has been reported to be associated with G2-phase cell cycle arrest [91]. Roles of S100 members on cell cycle control have further been supported by the fact that several of these protein members, namely S100A2 [85], S100A7 [92], S100A11 [90] and S100B [62] have been shown to modulate important molecules involved in cell cycle regulation, including CDKi, p21 and p27.

In addition, several of the S100 proteins, namely S100A2 [31], S100A4 [34], S100A6 [37, 61] and S100B [62, 93] have been shown to interact with p53, one of the key regulators of the cell cycle, with different functional effects. S100A2 has been shown to interact with the C-terminus of p53, thereby enhancing p53 induced transcriptional activity for *CDKN1A* (p21) [31]. Similarly, S100A6 has been reported to interact and stimulate p53 activity [37]. Conversely, interaction of S100A4 with the C-terminus of p53 has been shown to inhibit p53 dependent transcriptional activation of *CDKN1A* [34]. However, controversy exists regarding interpretation of the interactions between S100B and p53. S100B has been reported to co-operate with cPKC in regulating nuclear translocation of the wild type p53 thereby inducing p53-dependent transcriptional activation of *CDKN1A* and subsequent G1-phase cell cycle arrest [62]. On the other hand, S100B has also been demonstrated to inhibit the level of p53 and its transcriptional activity [93, 94]. In addition to S100 protein-mediated regulation of p53 function, p53 has been reported to regulate transcription of *S100A2* [95], *S100A6* [96] and *S100A9* genes [97]. Recently, *S100A14* has been identified as one of the transcriptional targets of p53 and this transcriptional regulation has been suggested to play a tumor suppressive role in esophageal squamous cell carcinoma [50]. Experimental evidence is, however, currently lacking to support these suggestions.

1.6. Tumor invasion and regulatory molecules: Role of MMPs

Tumor invasion and metastasis is one of the hallmarks of human cancer [13]. It is a complex process involving molecular alterations in cell adhesion molecules (CAMs) and ECM degrading proteases. CAMs consist of a variety of molecules like cadherins, integrins, selectins, immunoglobin superfamily and others like CD44. In addition to providing a mechanical link between cell to cell and cell to ECM, CAMs are actively involved in bidirectional (cell to ECM and ECM to cell) cell signaling [reviewed in 98 and 99]. E- and P-cadherins are expressed by keratinocytes and they provide cell-cell interactions and are also involved in intracellular signaling. Integrins represent the largest family of CAMs and they provide cell-ECM interactions. They are found as a heterodimer of α (16 different types) and β (8 different types) subunits and the different combinations of heterodimer recognize different ECM components. Once bound to their ligands, integrins mediate bidirectional signaling important for regulation of cell motility, cell survival and cell proliferation [12, reviewed in 100].

Proteolytic breakdown of cell-cell and cell-ECM interactions is necessary for invasion of tumor cells. Serine proteases and MMPs are the most important groups of proteases involved in tumor invasion and metastasis. MMPs are zinc-dependent endopeptidases consisting of more than 21 members which can cleave virtually any components of ECM [reviewed in 101]. By degrading ECM, MMPs not only allow cancer cells to invade into the stroma but also release other molecules (eg: growth factors) involved in cell growth, differentiation, apoptosis, angiogenesis and immune surveillance. Because of their involvement in multiple biological functions, the activity of MMPs is tightly regulated at various levels. In addition to transcriptional regulation, MMPs are synthesized as latent enzymes and need proteolytic cleavage for their activation. Moreover, endogenous tissue inhibitors (TIMPs) also regulate their activity [reviewed in 101]. However, cancer cells

acquire mechanisms to overcome such regulation and most of the metastatic cancers, including OSCCs, are often associated with increased expression and activity of several members of the MMPs [19, 20, 98, 102]. Accordingly, increased expression and activity of MMPs has been shown to be associated with increased metastatic potential and poor clinical outcomes in OSCCs [103, 104]. Previous work from our group identified amplification of chromosomal loci harbouring *MMP1*, *MMP3*, *MMP7*, *MMP8*, *MMP10*, *MMP12*, *MMP13* and *MMP20* genes and accordingly up-regulation of several of the MMP members in HNSCCs/OSCCs examined from different populations [17-20] (Suhr *et al*; unpublished data).

1.6.1. Role of S100 proteins in tumor invasion and metastasis

Several of the S100 protein members have been implicated in tumor invasion and metastases. Some of them have been found to correlate positively while some negatively with tumor invasion and metastases. Accordingly, several lines of experimental data have shown their involvement in key processes related to tumor invasion and metastasis (cytoskeletal organization and cellular motility) [27] and modulation of important molecules involved in cell adhesion and ECM degradation [46, 105]). Several *in vivo* and *in vitro* studies have linked over-expression of S100A4 (metastasin) with high invasion/metastatic potential and poor clinical outcomes in different human cancers [reviewed in 33, 105]. Metastasis promoting functions of the S100A4 have been related to its ability to modulate organization of cytoskeletal components [33], regulate expression of cell adhesion molecules (E-cadherin) [106] and regulate expression and activity of MMPs (MMP9 [107], MMP13 [108]). Functional association of other S100 members like S100A2 [109, 110], S100A6 [111], S100A8/A9 [112], S100A13 [42] and S100P [113] has also been demonstrated with tumor invasion. Recently, decreased immunoexpression of S100A14 has been shown to be correlated with high metastatic potential of colorectal cancers [43]. Nevertheless, the

functional significance and the molecular mechanisms of S100A14 in tumor invasion and metastasis are largely unknown.

2. Aims of the study

Previous work from our group, using high throughput genomic and proteomic methods, has identified differential expression of members of the S100 proteins in OSCCs/HNSCCs [18, 19]. These findings triggered further expression studies on *S100* gene members (**Paper I**) and investigation of the possible biological significance of one of the S100 members, in-particular S100A14, in OSCCs (**Papers II and III**).

General aims:

To identify differentially expressed *S100* gene family members in OSCCs and further to explore the possible functional role(s) and the associated molecular pathways related to S100A14 in OSCC progression.

Specific aims:

I. To investigate the differentially expressed *S100* gene family members (*S100A1, S100A2, S100A3, S100A4, S100A6, S100A7, S100A8, S100A9, S100A10, S100A11, S100A12, S100A13, S100A14, S100B, S100P* and *S100Z*) in OSCCs compared to their pair-wised normal controls and to correlate the findings with patients' clinicopathological parameters (**Paper I**).

II. To examine the expression pattern and sub-cellular localization of S100A14 in archival normal human oral mucosa (NHOM), oral dysplastic lesion (ODL) and OSCC tissue specimens and in an *in vitro* human OSCC progression model (**Papers II and III**).

III. To explore the biological role of S100A14 in regulation of OSCC derived cell proliferation and its functional association with the tumor suppressor protein p53 (**Paper II**).

IV. To investigate the functional role of S100A14 in regulation of OSCC cell invasion *in vitro* and to identify cell adhesion and invasion related molecules modulated by S100A14 (**Paper III**

3. Materials and methods

The materials and laboratory methods used in the study have been described in detail in the original papers included in the thesis. Therefore, a summary is presented here (**Figure 4**).

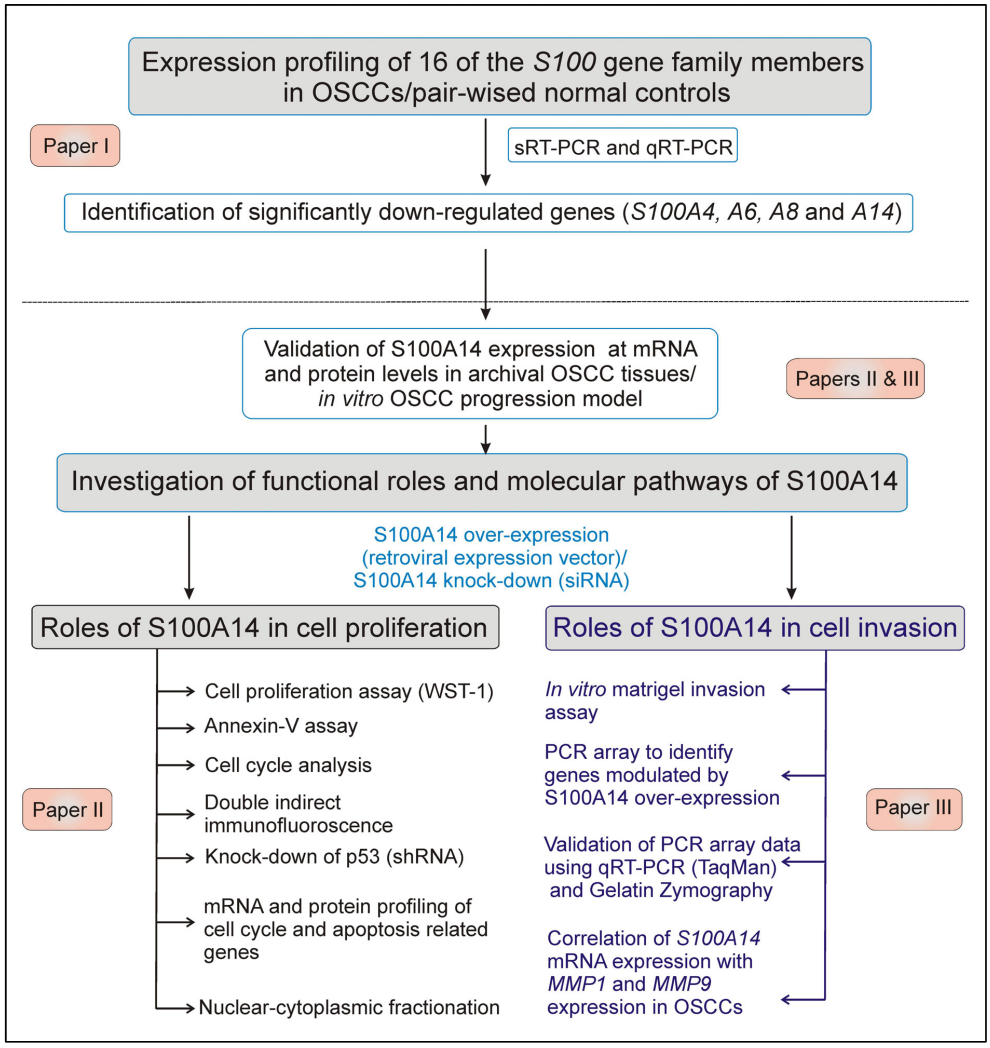

Figure 4. Flow chart illustrating the methodology used in the study

3.1. Patients, tissue specimens and cells

Both human tissue specimens and cells *(in vitro)* derived from normal, dysplastic and cancerous human oral epithelial tissues have been used in this study. NHOM tissues used to generate the keratinocyte primary culture and all other tissue specimens used in this study were obtained after written consent. This study was approved by the Regional Committees for Medical Ethics in Research.

3.1.1. Patients and tissue specimens

Table 3. Summary of the tissue specimens and the laboratory methods used in the study

Tissue specimens	Sample no.(*n*)	Procedures used	**Paper**
OSCCs/pair-wised normal controls (Sudan)	27	sRT-PCR[1] & qRT-PCR[2] for *S100* genes	**I**
OSCCs/pair-wised normal controls (Norway)	8	sRT-PCR & qRT-PCR for *S100* genes	**I**
NHOM, ODL and OSCCs (Sudan & Norway)	13, 10 and 16	IHC[3] for S100A14	**III**
OSCCs/pair-wised normal controls (Sudan)	19	qRT-PCR for *MMP1*, *MMP9* & *S100A14*	**III**

[1]semiquantitative RT-PCR; [2]quantitative RT-PCR; [3]immunohistochemistry

3.1.2. Cell culture (in vitro OSCC progression model)

An *in vitro* OSCC progression model consisting of cells derived from NHOM (NOK 94, NOK95, NOK108; *n*=3), ODL (POE9n [114] , D20 [115], DOK [116]; *n*=3) and oral carcinoma (SCC4 [117], SCC25 [117], H357 [118], VB6 [119], UK1 [120], CA1 [120], 5PT (T. Carey, University of Michigan), CaLH3 [121]; *n*=8) and OSCC tissues was used in the study (**Papers II and III**).

3.1.3. Selection of CaLH3 cell-line as an in vitro working model

Our main *in vitro* working model in **papers II and III** included the CaLH3 cell-line [121] derived from a primary OSCC located in the ventral surface of the tongue. We selected this cell-line for our work because of the following reasons:

I. It has satisfactory growth and handling characteristics.

II. It expresses an appreciable amount of S100A14 protein and this allows us to modulate (over-express or knock-down) the endogenous S100A14 expression according to the need of the experiment.

III. It harbors wild type (wt) p53 protein (exons 5 to 9 were sequenced) and this provides us with an opportunity to examine the possible involvement of p53 protein in S100A14 mediated functional effects.

3.2. mRNA and protein analyses

Several laboratory methods were used to profile expression of mRNA and protein levels and to investigate protein localization. mRNA levels were examined by sRT-PCR (**Paper I**) and qRT-PCR (**Papers I-III**). SYBR green based pathway focussed PCR Array (SABiosciences) was used to profile CAMs and ECM molecules (**Paper III**). Protein expression levels were examined by western blot (WB) analysis (**Papers II and III**). Proteolytic activity of MMP9 was examined by gelatine zymography (**Paper III**). Protein localization was examined by immunohistochemistry (IHC) (**Paper III**) and double indirect immunofluorescence (DIF) (**Paper II**). TaqMan assays used for qRT-PCR are summarized in **Table 4**. Details of the primary and secondary antibodies used for western blot, IHC and double indirect immunofluorescence are summarized in **Table 5**.

Table 4. Details of the TaqMan assays used in the study

Gene	Ref Seq	TaqMan assay ID	amplicon length (bp)
S100A1	NM_006271	Hs00196704_m1	74
S100A4	NM_002961	Hs00243201_m1	57
S100A6	NM_014624	Hs00170953_m1	94
S100A7	NM_002963	Hs00161488_m1	105
S100A8	NM_002964	Hs00374263_m1	70
S100A14	NM_020672	Hs00221080_m1	92
TP53	NM_001126112	Hs00153349_m1	72
Bcl-2	NM_000633	Hs00236808_s1	130
Bax	NM_138761	Hs00180269_m1	62
CDKN2A	NM_058195	Hs00923894_m1	115
CDKN1A	NM_078467	Hs00355782_m1	66
CDKN1B	NM_004064.3	Hs00153277_m1	71
MMP1	NM_002421	Hs00233958_m1	133
MMP9	NM_004994.2	Hs00957562_m1	67
FN1	NM_212474	Hs01549976_m1	81
CD44	NM_001001389	Hs00153304_m1	86

Table 5. Reagents and conditions used for WB, IHC and DIF.

Appl.	Target	Species	Catalog / Soruce	Dilution	Buffer*
WB	S100A14	P (rabbit)	10489-1-AP / Proteintech	1/1000	5% BSA / 1hr
WB	p21	M	556430 / BD pharmingen	1/200	5% BSA / 1hr
WB	p27	M	p2092/ DCS-72 / Sigma	1/200	5% BSA / 1hr
WB	p53	M	sc-263 / Santa Cruz	1/1000	5% BSA / 1hr
WB	p16	P (rabbit)	sc-468 / Santa Cruz	1/200	5% BSA / 1hr
WB	Bax	M	sc-20067 / Santa Cruz	1/1000	5% BSA / 1hr
WB	Bcl-2	P (rabbit)	sc-783 / Santa Cruz	1/500	5% BSA / 1hr
WB	GAPDH	M	ab 9484 / Abcam	1/5000	5% BSA / 1hr
WB	Lamin A/C	P (rabbit)	#2032 / Cell Signalling	1/1000	5% milk /ON[§]
WB	Anti-mouse	P (donkey)	715-035-150 / Jackson Immuno Research	1/1000	5% milk / 1hr
WB	Anti-rabbit	P (donkey)	711-035-152 / Jackson Immuno Research	1/1000	5% milk / 1hr
IHC	S100A14	P (rabbit)	10489-1-AP / Proteintech	1/500	AD / 1hr
IHC	Anti-rabbit	P (Goat)	DAKO - -		30 min
DIF	S100A14	P (rabbit)	10489-1-AP / Proteintech	1/1000	AD / 1hr
DIF	p53	M	DO-7 / DAKO	1/10	AD / 1hr, RT
DIF	Anti-mouse	P (goat)	Alexa Fluor 594 / Invitrogen	1/500	AD / 1hr, RT
DIF	Anti-rabbit	P (goat)	Alexa Fluor 488 / Invitrogen	1/500	AD / 1hr, RT

[*] TBST (Tris Buffered Saline, pH 7.4 with 0.1% Tween-20); [§] 4 °C, all other incubations at room temperature; Appl Applications; M monoclonal; P polyclonal; AD antibody diluent, DAKO; ON overnight

3.3. Selection of the endogenous control

RT-PCR is a highly sensitive laboratory technique. Hence, selection of an appropriate endogenous control is extremely important to normalize RT-PCR expression data. We tested 3 endogenous controls *(GAPDH, 18S* and *ACTB)* and *GAPDH* (with the lowest variation across the samples) was used to normalize both sRT-PCR and qRT-PCR data in **paper I**. Further, we tested 5 endogenous controls (*B2M, HPRT1, RPL13A, GAPDH* and *ACTB*) included in the PCR array across cell-lines derived from NHOM (NOK94, NOK95 and NOK108), ODL (D20 and DOK) and OSCCs (SCC4, SCC25). Their expression data were analyzed with NormFinder software (version 0.953) [122] and *GAPDH* with the lowest stability value (0.218) (the stability value is directly proportional to the variation in the expression status of the endogenous control gene across the samples) was used to normalize the data for all experiments (**Figure 5**).

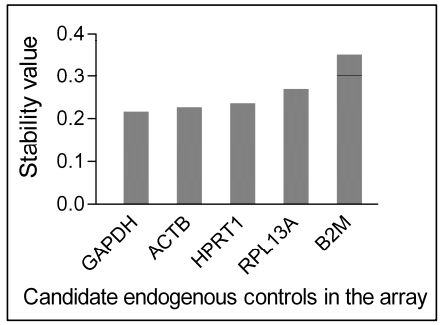

Figure 5. Five endogenous controls were examined using NormFinder software. *GAPDH*, followed by *ACTB*, were found to be the best working endogenous controls for gene expression profiling studies.

3.4. Modulation of S100A14 expression in vitro

The functional significance of S100A14 was examined by using retroviral vector mediated over-expression and siRNA mediated knock-down of the endogenous S100A14. Following successful over-expression and knock-down of the S100A14, *in vitro* assays were carried out. CaLH3 and H357 cells infected with retrovirus with S100A14 insert and retrovirus without

S100A14 insert are referred to as 'S100A14-CaLH3 and S100A14-H357' and 'control-CaLH3 and control-H357', respectively. Similarly, CaLH3 cells treated with scrambled and S100A14 siRNA are referred to as 'sc-siRNA' and 'S100A14-siRNA' cells, respectively.

S100A14 shares significant similarity with other members of the S100 family. Thus specificity of S100A14 retroviral expression vector and S100A14 siRNA was verified by examining mRNA expression of other *S100* gene members related to S100A14, namely *S100A13, S100A4, S100A10* and *S100A9*. mRNA expression levels of these S100A14 related genes were found to be unaltered both in control and treated CaLH3 and H357 cell-lines, suggesting high specificity of the S100A14 retroviral expression vector and S100A14 siRNA used.

3.5. Functional assays

Effects of S100A14 over-expression/knock-down on cell proliferation (WST-1 assay), apoptosis (Annexin-V assay), cell cycle kinetics (Propidium Iodide staining and flow cytometry) and cell invasion (Matrigel invasion assay) were examined using established *in vitro* methods.

4. Summary of the results and discussion

I. Differential expression of the members of the S100 gene family in OSCCs: down-regulation of S100A14 in OSCC specimens and OSCC derived cell-line in vitro (Papers I-III)

Both genotypic and phenotypic alterations characterize malignant transformation of normal cells. Among the different genetic alterations found in human cancers, increasing numbers of the *S100* gene family members have been described in connection with tumorigenesis because of their differential expression found in human malignancies and their association with several key biological functions. In this study, a comprehensive expression profiling of 16 of the *S100* gene family members (*S100A1, S100A2, S100A3, S100A4, S100A6, S100A7, S100A8, S100A9, S100A10, S100A11, S100A12, S100A13, S100A14, S100B, S100P and S100Z*) using sRT-PCR and further verification with qRT-PCR revealed altered mRNA expression of several of the *S100* gene members examined in OSCCs. Four of the *S100* members (*S100A4, S100A6, S100A8* and *S10014)* were found to be significantly down-regulated ($p<0.05$) in the OSCCs ($n=27$) examined from the Sudan compared to their pair-wised normal controls (**Figure 6A**). *S100A14* was also found to be down-regulated in the OSCC cases examined from Norway ($n=8$) ($p=0.015$) (**Figure 6B**). Similar to these findings, altered expression of *S100* gene family members has been reported in several human cancers [reviewed in 46, 52, 123] suggesting their association with human carcinogenesis. Though the exact mechanism for differential expression of *S100* gene members in human cancers is largely unknown, yet several possible explanations have been proposed. Firstly, frequent structural and numerical aberrations in the chromosomal region 1q21 [45, 124] (where most of the S100 members are located [24]) have been reported in a variety of human cancers.

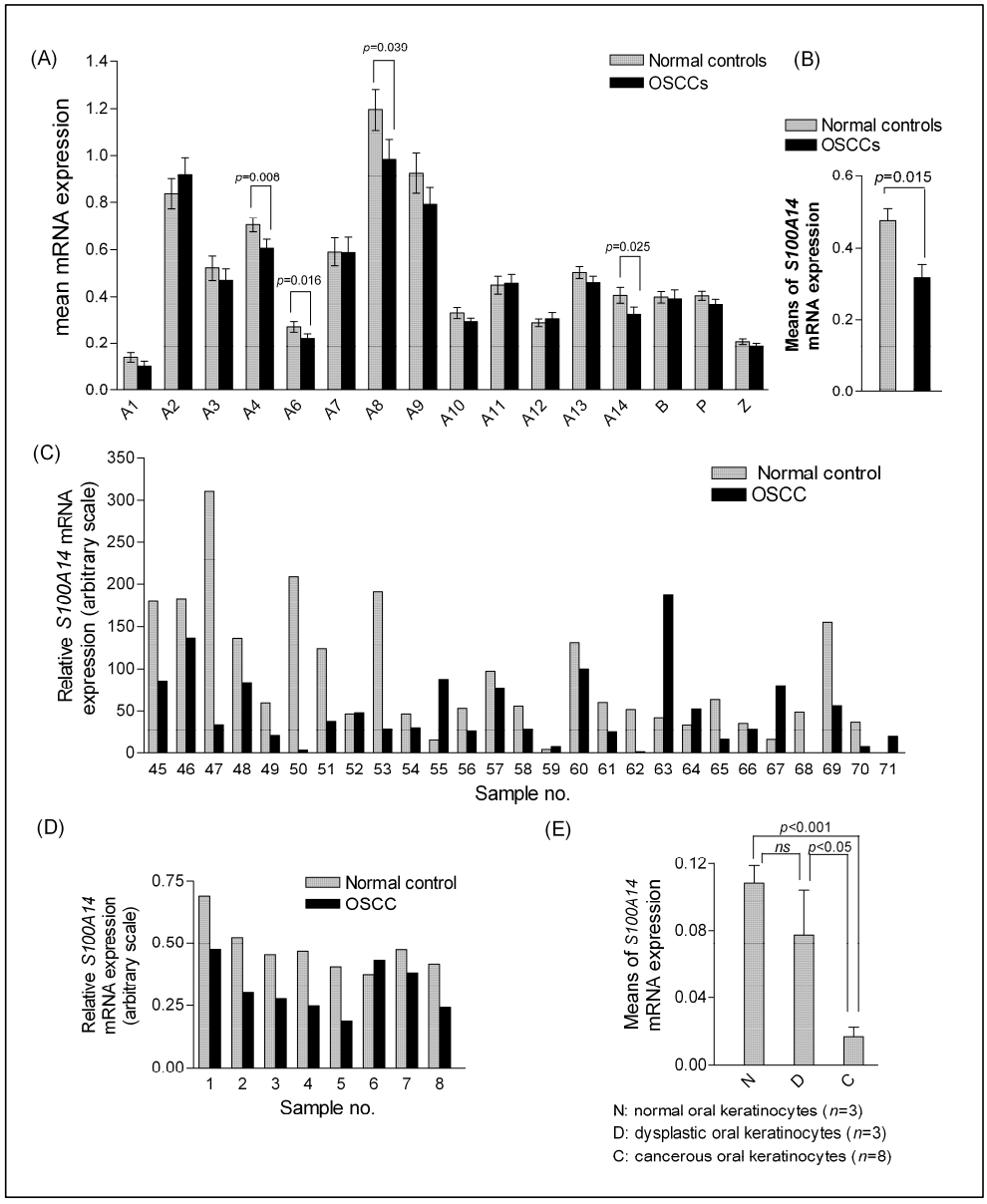

Figure 6. Means of the mRNA expression levels of 16 *S100* gene members examined in OSCCs from Sudan (A). Mean of the *S100A14* mRNA expression in OSCCs from Norway (B). Error bars in figures (A) and (B) represent standard error of the mean (SEM). Distribution of *S100A14* mRNA in OSCCs and their pair-wised normal controls from Sudan (C) and Norway (D). (E) Means of *S100A14* mRNA expression levels in the *in vitro* OSCC progression model. Error bars represent standard deviation (SD).

Supporting these observations, previous studies from our group have shown chromosomal rearrangements in the 1q21 region harbouring most of the *S100* gene members in HNSCCs/OSCCs examined from different populations [17] (Suhr *et al*; unpublished observations). These findings indicate that chromosomal rearrangements might be one of the mechanisms for the differential expression of the *S100* gene members in the OSCC cases examined. Secondly, it has been shown that the chromosome region 1q21-1q22 contains extended regions of high CpG island density [125]. The methylation status of CpG islands in the promoter region has been shown to regulate the transcription of genes. Interestingly, differential expression of many *S100* gene members in different cancer types has been shown to be associated with the methylation status of CpG islands in *S100* genes [126-130]. Therefore, it is possible that the down-regulated expression of *S100* gene members found in OSCCs might also be due to transcription repression by hypermethylation of CpG islands. This suggestion, however, requires further investigation.

Mirroring down-regulated expression of the *S100A14* mRNA in Sudanese and Norwegian OSCC cases (**Figure 6C** and **D**), S100A14 immunoexpression was found to be weaker in the OSCCs; especially in the invading islands of tumor cells (**Figure 7**; **Figure 1** in **Paper III**). These findings were paralleled by the *in vitro* data where *S100A14* mRNA (**Figure 6E**) and protein (**Figure 1** in **Paper II**) levels were found to be down-regulated in the OSCC derived cell-lines compared to that of the normal oral keratinocytes. These observations indicate that loss of S100A14 is closely associated with OSCC progression. In addition, *S100A14* mRNA and protein levels were found to be altered in the dysplastic cell-lines compared to the normal oral keratinocytes *in vitro* (**Figure 6E**; **Figure 1** in **Paper II**), suggesting that de-regulation of S100A14 might be an early event in OSCC carcinogenesis. Immunohistochemical analysis of S100A14 in ODL (n=10), although showed a heterogeneous expression pattern with some ODL displaying weaker and some showing

stronger S100A14 staining compared to that of the NHOM, supports the notion that altered expression of S100A14 might be an early molecular event in OSCC carcinogenesis. Nevertheless, further investigation using a large cohort of specimens is warranted to confirm these findings and to examine the possibility of 'translational relevance' of the expression pattern of S100A14 in ODL.

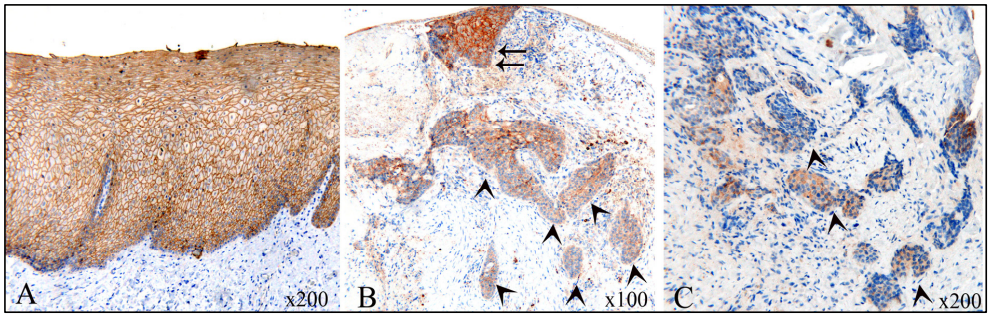

Figure 7. Expression and sub-cellular localization of the S100A14 in NHOM and OSCC as examined by IHC. Representative NHOM (A) specimen showing strong membranous S100A14 expression in the epithelial compartment. (B) OSCC lesion showing a gradient of S100A14 immunostaining- remains of the superficial epithelium (arrows) shows a strong, membranous staining in contrast to a weak, predominantly cytoplasmic staining in the tumor invading islands (arrowheads).(C) High magnification of the OSCC lesion demonstrating weak, predominantly cytoplasmic S100A14 staining in the tumor invading islands (arrowheads).

Down-regulation of *S100A14* mRNA and protein levels found in the OSCCs and OSCC derived cell-lines is in agreement with previous reports in different human cancers [43, 52]. Strong membranous S100A14 staining in NHOM compared to weak membranous and cytoplasmic staining in the invading tumor islands found in OSCC is in accordance with a previous report in colorectal carcinomas [43]. However, in their study the authors did not describe any change in sub-cellular localization in cancers compared to normal colorectal tissues in their study. A change in sub-cellular localization of S100A14 from membranous in the highly differentiated areas to cytoplasmic in the poorly differentiated areas has been described in tonsil squamous cell carcinoma and bladder papillary transitional cell carcinoma

[49]. This is in agreement with our observations that the invading islands of cells (poorly differentiated) showed mixed membranous and cytoplasmic or cytoplasmic staining compared to the membranous staining found in more differentiated (central/superficial) areas in OSCC and NHOM tissue specimens (**Figure 1** in **Paper III**). These data suggest that membranous S100A14 might play a role in establishing cell-cell contacts in non-invading cells and that this function might be lost in the invading cancer cells perhaps due to membrane to cytoplasmic switch of S100A14 sub-cellular localization. However, this suggestion needs to be rigorously tested. In contrary, an opposite S100A14 staining pattern and sub-cellular localization has been claimed in breast cancers [49]. Nevertheless, only 17% (10 out of 58) of the breast cancers examined have been described to express strong S100A14 immunoreactivity in their study and this implies that 83% of the cases were perhaps weaker in S100A14 expression [49]. Nonetheless, the differences in the expression and sub-cellular localization of S100A14 might be due to differences in the source of the archival tissues used (oral vs. breast), indicating the possibility of a context dependent regulation of S100A14 expression, or due to the procedural differences in IHC (among others, we have used Tris-EDTA, pH9 and while the authors have used Citrate buffer, pH6 supplemented with 1mg/mL pepsin treatment for antigen retrieval).

Down-regulation of the S100A14 was found at the transcriptional level in the tissue samples of OSCC and in the OSCC derived cell-lines. This could be due to rearrangement of the *S100A14* locus as evidenced by a high degree of chromosomal rearrangement of the 1q21 region found in HNSCCs/OSCCs [17] (Suhr *et al*; unpublished observations). Conversely, no deletions or gross rearrangement have been reported for the *S100A14* gene in lung cancer cell-lines [26]. This discrepancy could be due to different genetic alterations found in different tissues of origin (oral vs. lung) and therefore this finding cannot completely rule out the correlation of down-regulated expression of S100A14 in OSCCs with the frequently observed rearrangements in 1q21 region found in HNSCC and other human cancers. On the other hand,

hypermethylation of CpG islands is a very unlikely mechanism for this down-regulation since no CpG islands have been reported in the *S100A14* gene [26]. In addition, sequences upstream of the transcription initiation site of *S100A14* have been suggested to contain consensus recognition sequences for a number of transcriptional factors like c-Myc/Max, AREB6, USF and E2 box repressor deltaEF1; and negative regulatory elements like TCF11-, NF-kB-, c-Ets1-, MZF1-, and estrogen receptor-binding sites [26]. These observations further suggest the possible existence of other regulatory mechanisms for *S100A14* expression and indicate S100A14 to be a major player in a complex molecular interplay involved in biological processes.

Altered expression of many S100 members has been described in connection with different aspects of tumorigenesis, like cell proliferation and apoptosis [85, 87, 89-91, 131, 132], tumor invasion and metastasis [reviewed in 105, 106, 110, 111]. In addition, different sub-cellular localization of S100A6 and S100A11 proteins have been described to be associated with cell proliferation, tumor progression and poor clinical outcomes [90, 133-135]. Hence, both quantitative (expression) and qualitative (sub-cellular localization) changes of S100A14 expression might be associated with change/alteration in the function of this protein and that might be important in the OSCC tumorigenesis process.

II. S100A14 over-expression mediated regulation of cell cycle and cell proliferation (Paper II)

One of the hallmarks of cancer is uncontrolled tumor growth [13]. The key determinants of tumor growth are rate of cell proliferation (cell division) and cell death (apoptosis). It is obvious that abnormalities in the mechanisms controlling cell proliferation and/or apoptosis might lead to excessive cellular growth. Cancer cells frequently acquire genetic defects (abilities) to escape these mechanisms and proliferate in an uncontrolled fashion. We found

down-regulation of S100A14 both in OSCC specimens and in OSCC derived cell-lines, indicating that loss of S100A14 might be related to the OSCC progression.

Examination of the cellular proliferation showed that CaLH3 cells proliferated significantly slower when S100A14 was over-expressed by employing retroviral expression vector. In addition, higher fraction of S100A14-CaLH3 cells was found to be arrested in the G1-phase of cell cycle (**Figure 3** in **Paper II**). Taken together, these data indicate that S100A14 over-expression inhibits proliferation of S100A14-CaLH3 cells by inducing G1-phase cell cycle arrest. Further, over-expression and siRNA mediated knock-down of S100A14 was found to be associated with up-and down-regulation of the CDKi p21 and p27 in CaLH3 cells (**Figure 3** in **Paper II**). These findings imply that p21 and p27 proteins function downstream of the S100A14 protein and this functional association is related to the observed G1-phase cell cycle arrest in S100A14-CaLH3 cells. It is therefore possible that loss of S100A14 expression in OSCCs might enable tumor cells to escape S100A14 mediated control over the cell cycle, thus allowing them to proliferate excessively.

III. S100A14 over-expression mediated regulation of p53 activity and function (Paper II)

The observations that (i) several members of the S100 protein family interact with p53 both with stimulating as well as inhibiting effects on p53 functions relevant for human carcinogenesis [31, 34, 37, 61, 93, 94] (ii) S100A14 over-expression induced G1-phase cell cycle arrest with up-regulation of p21 (one of the key transcriptional targets of p53) in CaLH3 cell-line; led us to investigate the possible involvement of p53 in the S100A14 mediated functional effect on the cell cycle. shRNA mediated knock-down of p53 resulted in suppression of p21 expression in S100A14-CaLH3 cells, indicating that S100A14 over-expression mediated p21 up-regulation was indeed dependent on the activity of p53 (**Figure 3** in **Paper II**). Supporting these findings, inhibition of cell proliferation, induction of G1-phase

cell cycle arrest or up-regulation of p21 protein was not found by over-expressing S100A14 in the H357 cell-line harboring a mutated and non-functional p53 (**Figure 5** in **Paper II**).

Examination of the *TP53* mRNA, total and nuclear-cytoplasmic p53 protein analyses showed that S100A14 over-expression was associated with nuclear accumulation of p53, without any effect on the transcription of *TP53*; indicating that S100A14 over-expression enhances stability and subsequent nuclear accumulation of p53 (**Figure 4** in **Paper II**). Since nuclear accumulation of p53 is considered as one of the major mechanisms to enhance p53 activity [60, 136], we propose that S100A14 over-expression promotes p53 activity by favoring p53 nuclear accumulation and that might be related with the p21 up-regulation and the G1-cell cycle arrest in CaLH3 cells. In fact, modulation of p53 activity by favoring p53 nuclear accumulation has been suggested for other members of the S100 proteins, such as S100A6 [37] and S100B [62].

Chen *et al* have recently shown that *S100A14* gene is a transcriptional target of p53 [50]. Given the fact that S100A14 regulates nuclear accumulation and activity of p53 as shown in the current study, it is possible that there exists a mutual positive functional regulation between p53 and S100A14 and that might be important in the tumor suppressive functions involved in OSCC carcinogenesis. Such mutual regulation between S100A2 and p53 has already been suggested [31, 95].

IV. S100A14 over-expression mediated regulation of tumor cell invasion through modulation of MMP1 and MMP9 expression and activity (Paper III)

OSCC is a highly aggressive pathological condition characterized by frequent metastatic involvement of the cervical lymph nodes, resulting in a severely reduced patient survival [137, 138]. A cascade of qualitative and quantitative molecular (intracellular, intercellular and cell-ECM) alterations influences the invasive potential of cancer cells and also modulates the ECM microenvironment to support the motility of cancer cells. Our findings of (i)

weak/absent S100A14 immunoexpression in the invading islands of tumor cells compared to the more superficial areas in OSCCs (ii) membrane to cytoplasmic switch of the S100A14 sub-cellular localization in the invading islands of tumor cells in OSCCs (iii) weak/absent *S100A14* mRNA expression in invasive VB6, H357 and SCC25 oral carcinoma derived cell-lines; suggest that S100A14 expression might be associated with the invasive phenotype of OSCC. This idea has further been substantiated by previous reports where reduced expression of S100A14 has been correlated with the increased metastatic potential in colorectal carcinomas [43]. Moreover, a change in sub-cellular localization of the S100A14 from membranous in more differentiated (central) areas to cytoplasmic in poorly differentiated (invading) areas in tonsil squamous cell carcinoma and bladder papillary transitional cell carcinoma has also been reported [49].

Corroborating the observed correlation between loss of S100A14 expression and invasive phenotype of the cells in OSCC specimens and in the OSCC derived cell-lines, a functional role of S100A14 in tumor cell invasion was evidenced by the fact that over-expression of endogenous S100A14 inhibited the invasive potential of CaLH3 (P=0.039) and H357 (P=0.066) cell-lines when using quantitative *in vitro* Matrigel invasion assay (**Figure 4** in **Paper III**). Supporting these findings, siRNA mediated knock-down of the S100A14 promoted invasion of CaLH3 cells (P=0.011) (**Figure 4** in **Paper III**). Collectively, these findings indicate that expression of S100A14 is negatively related with the invasive phenotype of tumor cells and loss of S100A14 expression in tumor cells might therefore contribute to the more invasive phenotype of these cells.

Unsupervised hierarchical cluster analysis of the PCR array data showed a distinct mRNA signature of the genes involved in tumor invasion and metastasis in control-CaLH3 cells compared to the S100A14-CaLH3 cells. These observations indicate that S100A4 over-expression mediated change in the invasive phenotype of S100A14-CaLH3 cells was indeed

related to the modulation of these invasion and metastasis related genes. In accordance, SAM analysis identified a number of invasion and metastasis promoting (*MMP1, MMP9, FN1, CD44* and *TNC)* and suppressing genes *(TIMP2)* to be significantly (FDR=0) down- and up-regulated, respectively, in S100A14-CaLH3 cells. S100A14-CaLH3 cells expressed 12.63 and 8.38 folds less *MMP1* and *MMP9* mRNAs compared to that of control-CaLH3 cells. In parallel, *MMP1* and *MMP9* mRNA levels were also found to be suppressed in S100A14-H357 cells compared to the control-H357 cells (**Figure 5** in **Paper III**). Additionally, activity of MMP9 was found to be suppressed in S100A14-CaLH3 cells (**Figure 5** in **Paper III**). Overall, these data suggest that *MMP9* and *MMP1* are among the key targets of S100A14 and suppression of their expression and activity might contribute to the S100A14 over-expression mediated reduction of the invasive potential of tumor cells. These suggestions were also reflected in the OSCCs specimens where *MMP1* and *MMP9* mRNA expression levels were found to be inversely correlated with the *S100A14* mRNA level. Given the fact that MMP9 and MMP1 are frequently over-expressed and are associated with increased metastatic potential and poor clinical outcomes in OSCCs [139-142], it is possible that one way the transformed cells can acquire an invasive phenotype might be through the up-regulation of *MMP9* and *MMP1* by down-regulating S100A14 expression.

In conclusion, these data propose that S100A14 regulates the invasive phenotype of OSCC derived cells by modulating expression and activity of MMP1 and MMP9. Down-regulation of S100A14 found in OSCCs might be associated with the increased invasive and metastasis potential of the tumor cells and that might explain a high rate of cervical lymph node metastasis found in these lesions.

5. Conclusions

A comprehensive mRNA expression profiling of 16 of the *S100* gene members in OSCCs was carried out and functional roles and related molecular networks were characterized for the S100A14. The following conclusions can be drawn based on the data of the current study:

I. Differential expression of *S100* gene members is a common molecular change found in the OSCCs examined, indicating a close association of these gene members with oral carcinogenesis.

II. Down-regulation of S100A14 is a common molecular alteration observed in OSCCs, highlighting its role in progression of OSCC.

III. S100A14 mediated inhibition of OSCC cell proliferation due to G1- arrest through nuclear accumulation of p53 and p53-dependent up-regulation of p21 indicates that the functional relation between S100A14 and p53 plays a tumor suppressive role in OSCC carcinogenesis.

IV. S100A14 over-expression mediated inhibition of OSCC cell invasion with concomitant suppression of MMP1 and MMP9 expression and activity suggest that down-regulation of S100A14 found in OSCCs might be related to increased invasive and metastasis potential of these lesions.

V. Down-regulated expression of S100A14 in OSCCs and its involvement in the inhibition of cell proliferation and invasion of OSCC derived cells suggests that S100A14 might function as a tumor suppressive protein in OSCC carcinogenesis (**Figure 8**).

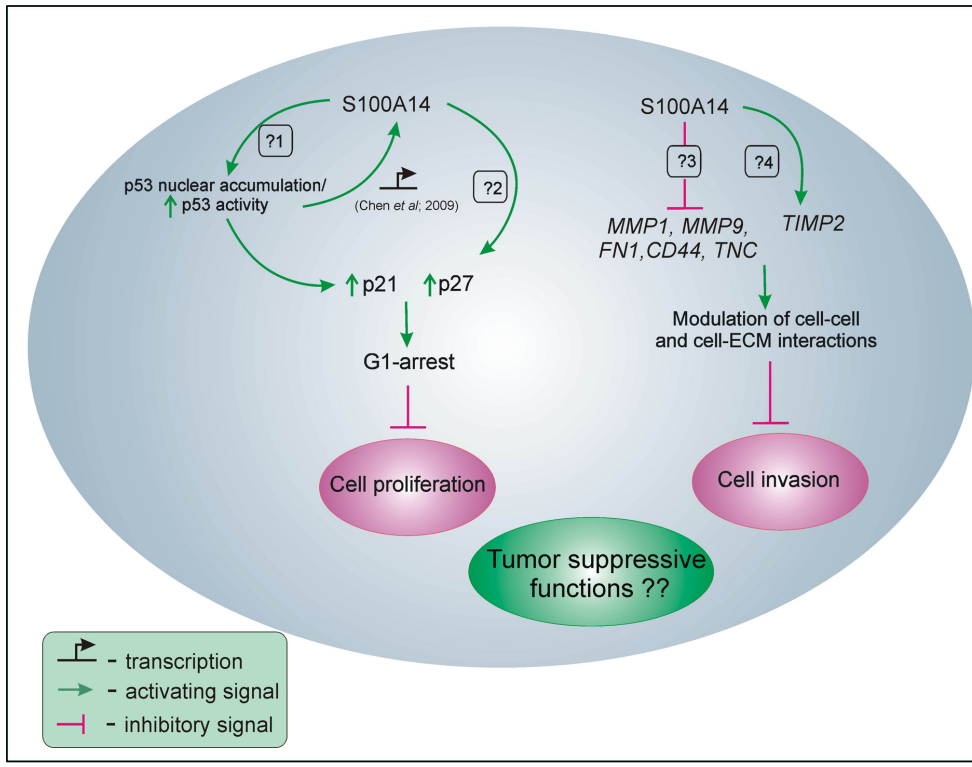

Figure 8. Involvement of S100A14 in cell proliferation and cell invasion, suggesting a tumor suppressive role for S100A14. Question mark 1 (?1) indicates unknown mechanism associated with nuclear accumulation of p53. ?2,?3 and ?4 illustrate unknown mechanisms involved in the regulation of the indicated molecules.

6. Future perspectives

Further investigations are necessary to confirm and to extend the findings of the current study employing additional *in vitro* and *in vivo* model systems.

I. Regulation of the expression of S100A14 in OSCCs

Down-regulation of S100A14 was found in OSCCs and in OSCC derived cell-lines. Although some of the possible mechanisms for this down-regulation have been discussed, further investigations will be necessary to fully understand the molecular mechanisms involved in S100A14 expression.

II. Signalling between S100A14, p53 and p27

This study provided evidence that S100A14 over-expression mediated up-regulation of p21 is p53-dependent. However, the molecular mechanisms related to nuclear accumulation of p53 and up-regulation of p27 associated with S100A14 over-expression need to be examined.

III. S100A14 mediated modulation of identified invasion and metastasis related genes, in particular MMP1 and MMP9

Some of the molecular mechanisms (nucleobindin mediated G protein-coupled signal transduction, **Paper III**) possibly involved in the modulation of mRNA expression of *MMP1* and *MMP9* and other invasion and metastasis related genes associated with S100A14 over-expression have been discussed in the current work. However, this possibility needs to be rigorously established.

IV. Significance of membrane to cytoplasmic switch of S100A14 in invading islands of tumor cells

The functional and clinical significance and molecular mechanisms associated with membrane to cytoplasmic switch of S100A14 protein in OSCCs need to be elucidated.

V. Translational relevance

Altered expression of S100A14 was noticed even in the dysplastic stage. In addition, findings such as loss of S100A14 in invasive islands of tumor cells with membrane to cytoplasmic switch strongly indicate association of the S100A14 expression pattern with the progression of disease and its severity. These associations/correlations, though not rigorously established in this study definitely warrant further investigation, and represent promising variables that might be useful in clinical settings.

7. References

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