Clinical Review

Calprotectin and the Initiation and Progression of Head and Neck Cancer

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P.P. Argyris[1], Z.M. Slama[1], K.F. Ross[1], A. Khammanivong[2,3], and M.C. Herzberg[1]

Abstract

Calprotectin (S100A8/A9), a heterodimeric complex of calcium-binding proteins S100A8 and S100A9, is encoded by genes mapping to the chromosomal locus 1q21.3 of the epidermal differentiation complex. Whereas extracellular calprotectin shows proinflammatory and antimicrobial properties by signaling through RAGE and TLR4, intracytoplasmic S100A8/A9 appears to be important for cellular development, maintenance, and survival. S100A8/A9 is constitutively expressed in myeloid cells and the stratified mucosal epithelia lining the oropharyngeal and genitourinary mucosae. While upregulated in adenocarcinomas and other cancers, calprotectin mRNA and protein levels decline in head and neck squamous cell carcinoma (HNSCC). S100A8/A9 is also lost during head and neck preneoplasia (dysplasia). Calprotectin decrease does not correlate with the clinical stage (TNM) of HNSCC. When expressed in carcinoma cells, S100A8/A9 downregulates matrix metalloproteinase 2 expression and inhibits invasion and migration in vitro. S100A8/A9 regulates cell cycle progression and decelerates cancer cell proliferation by arresting at the G2/M checkpoint in a protein phosphatase 2α–dependent manner. In HNSCC, *S100A8* and *S100A9* coregulate with gene networks controlling cellular development and differentiation, cell-to-cell signaling, and cell morphology, while S100A8/A9 appears to downregulate expression of invasion- and tumorigenesis-associated genes. Indeed, tumor formation capacity is attenuated in S100A8/A9-expressing carcinoma cells in vivo. Hence, intracellular calprotectin appears to function as a tumor suppressor in head and neck carcinogenesis. When compared with S100A8/A9-low HNSCC based on analysis of TCGA, S100A8/A9-high HNSCC shows significant upregulation of apoptosis-related genes, including multiple caspases. Accordingly, S100A8/A9 facilitates DNA damage responses in HNSCC, promotes apoptotic cell death, and confers sensitivity to cisplatin and X-radiation in vitro. In the tumor milieu, loss of S100A8/A9 strongly associates with poor squamous differentiation and higher tumor grading, EGFR upregulation, increased DNA methylation, and, finally, poorer overall survival for patients with HNSCC. Hence, intracellular calprotectin shows a multifaceted protective role against the development of HNSCC.

Keywords: S100A8/A9, oral squamous cell carcinoma, oropharyngeal squamous cell carcinoma, esophageal squamous cell carcinoma, nasopharyngeal squamous cell carcinoma, thyroid adenocarcinoma

Role of Calprotectin in Cellular Functions

Calprotectin (S100A8/A9) belongs to the S100 superfamily of EF-hand calcium-binding proteins, which contains >20 members (Donato 2001; Itou et al. 2002). Formed as a heterodimeric protein complex of S100A8 (MRP8 or calgranulin A; 8 kDa) and S100A9 (MRP14 or calgranulin B; 14 kDa) and encoded by genes that map to the human epidermal differentiation complex (EDC) on chromosomal locus 1q21.3, calprotectin is implicated in calcium-dependent regulation of cellular differentiation, proliferation, motility, and gene expression (Donato 2001; Itou et al. 2002). Genes located within the EDC on chromosome 1q21, including S100A8 and S100A9, are crucial to maintain normal epithelial phenotype, tissue development, and repair (Kypriotou et al. 2012; Abhishek and Palamadai Krishnan 2016). As a part of the EDC, S100A8/A9 and other proteins appear to regulate epithelial maturation, differentiation, and growth (Hsu et al. 2009).

S100A8/A9 is also essential for myeloid cell differentiation. S100A8, S100A9 and calprotectin complex are expressed during early stages of differentiation and cellular infiltration and are involved in regulation of casein kinase I and II (Donato 2001; Hessian and Fisher 2001; Bhattacharya et al. 2004). Later in development, S100A8/A9 is typically produced and released by infiltrating cells of the immune system, including polymorphonuclear leukocytes and macrophages, secretory cells, and damaged epithelial cells (Hessian and Fisher 2001; Hsu et al. 2009). Like polymorphonuclear leukocytes, normal mucosal squamous epithelial cells constitutively express S100A8/A9 in the cytoplasm (Bhattacharya et al. 2004). Within epithelial cells, calprotectin can activate NADPH oxidase to generate antibacterial, reactive-oxygen species and

[1]Department of Diagnostic and Biological Sciences, School of Dentistry, University of Minnesota, Minneapolis, MN, USA
[2]Department of Veterinary Clinical Sciences, University of Minnesota, St. Paul, MN, USA
[3]Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA

Corresponding Author:
M.C. Herzberg, Department of Diagnostic and Biological Sciences, School of Dentistry, University of Minnesota, 17-164 Moos Tower, 515 Delaware St. SE, Minneapolis, MN 55455, USA.
Email: mcherzb@umn.edu

activate nuclear factor-kappaB (NF-κB) signaling (Benedyk et al. 2007; Berthier et al. 2012). Activation of NADPH oxidase appears to depend on binding of arachidonic acid at the HHH domain of the C-terminal region of S100A9 and phosphorylation at Thr113 (Fig. 1; Benedyk et al. 2007).

During acute and chronic inflammatory responses, released calprotectin functions as an innate immune response regulatory or effector molecule (Iotzova-Weiss et al. 2015; Narumi et al. 2015). In the extracellular environment, calprotectin represents a key antimicrobial protein found in neutrophil extracellular traps (Urban et al. 2009). Extracellular S100A8/A9 is a biomarker of inflammatory disorders such periodontitis and inflammatory bowel disease (Kido et al. 2004; Baldassarre et al. 2007). Functioning as an intracellular antimicrobial protein, S100A8/A9 enhances epithelial cell resistance to invasion by oral and enteric bacterial pathogens, including *Porphyromonas gingivalis, Salmonella Typhimurium*, and *Listeria monocytogenes* (Nisapakultorn et al. 2001; Champaiboon et al. 2009).

S100A8/A9 in tissue spaces is considered an "alarmin," signaling a proinflammatory response through the receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4). By engaging calprotectin, TLR4 signaling amplifies innate inflammatory responses, including that associated with solid tumors (Ehrchen et al. 2009). RAGE is a cell surface molecule representing a multiligand receptor of the immunoglobulin superfamily. Binding of RAGE by S100A8/A9 triggers the activation of downstream cellular pathways, including mitogen-activated protein kinases (MAPKs), Cdc42/Rac, and NF-κB signaling pathways, which control cell survival, cell motility, and inflammatory responses (Taguchi et al. 2000; Hermani et al. 2006). Released calprotectin may act as a chemotactic agent and contribute to the recruitment of human monocytes and granulocytes to sites of inflammation (Eue et al. 2000). In septic patients, S100A8/A9 and RAGE are significantly elevated in peripheral blood leukocytes (Hofer et al. 2016)

Our understanding about how intracellular calprotectin may contribute to carcinogenesis is hitherto limited. In stratified squamous epithelium lining the oral mucosa, calprotectin expression is higher in the superficial spinous and keratin cell layers; in the deeper suprabasal and basal cells, detection is absent (Martinsson et al. 2005; Funk et al. 2015). Hence, calprotectin appears to contribute to differentiation and maturation of oral keratinocytes in epithelia. Consistent with a role in epithelial maturation and renewal, S100A8/A9 induces autophagy and apoptosis (Ghavami et al. 2004; Ghavami et al. 2008) and significantly increases caspase 3/caspase 7 activity in multiple human cell types (Ghavami et al. 2010). In prostate carcinoma cells, calprotectin appears to induce cell death by downregulating the antiapoptotic protein survivin and increasing reactive-oxygen species (Sattari et al. 2014). S100A9 alone promoted apoptosis and compromised growth of acute promyelocytic leukemia cells (Zhu et al. 2017). Similarly, treatment of cervical squamous cell carcinoma cells (CaSki cells) with purified recombinant S100A8 and S100A9 proteins also induced apoptosis and inhibited cell migration (Qin et al. 2010), suggesting that released calprotectin can function in an autocrine manner. Figure 1 summarizes the putative structure and functional properties of the S100A8/A9 complex.

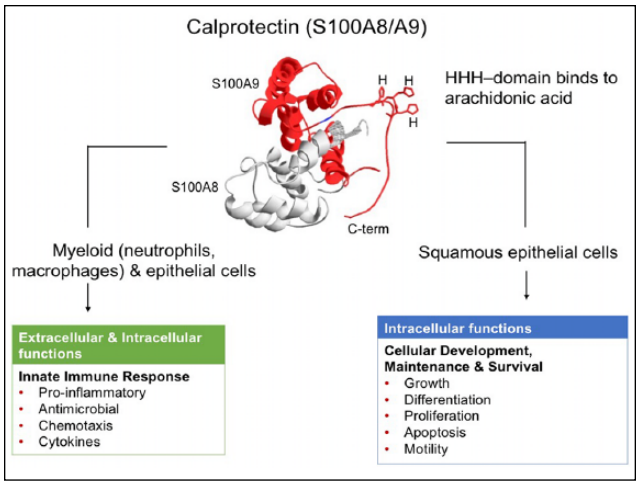

Figure 1. Putative structure and functions of calprotectin (S100A8/A9). HHH-domain binds arachidonic acid, as indicated in the C-terminal region (C-term) of the S100A9 subunit of the calprotectin complex.

Expression and Tissue Distribution of S100A8/A9 in Human Cancer

During carcinogenesis, inflammatory cells release S100A8/A9 into the tumor microenvironment (Gebhardt et al. 2002; Gebhardt et al. 2006). Extracellular calprotectin may contribute to inflammation-induced tumor initiation and progression (Gebhardt et al. 2002). In human malignancies—including prostate, gastric, colorectal, breast, bladder, and oropharyngeal carcinomas—S100A8/A9 expression levels in neoplastic cells and/or tumor-associated macrophages may reflect prospects for the progression of the disease and patient overall survival (Yao et al. 2007; Fan, Zhang, et al. 2012; Kim et al. 2014; Tidehag et al. 2014; Funk et al. 2015; Bao et al. 2016; Khammanivong et al. 2016; Moris et al. 2016).

Calprotectin is expressed in a cell- and tissue-specific manner in mature and differentiating normal tissues (Donato 2003). Expression of S100A8 and S100A9 can also be influenced by epigenetic factors. Normal human epithelial tissues that do not endogenously express calprotectin include skin (Gebhardt et al. 2006), breast (Moon et al. 2008; Rodriguez-Barrueco et al. 2015), thyroid (Ito et al. 2005; Ito et al. 2009), liver (Németh et al. 2009), gastric mucosa (Turovskaya et al. 2008; Fan, Zhang, et al. 2012), prostate (Hermani et al. 2006), ovary (Ødegaard et al. 2008), bladder (Yao et al. 2007), and lung (Arai et al. 2001). In primary malignant neoplasms derived from these tissues, primarily adenocarcinomas, S100A8/A9 expression is generally induced (Fig. 2, red arrow). Whether increased calprotectin expression observed in these malignant neoplasms is a sequela of carcinogenesis or actually drives tumor development and progression is unclear.

In contrast, S100A8/A9 is constitutively expressed in normal mucosal tissues lined by stratified squamous epithelia (Gonzalez

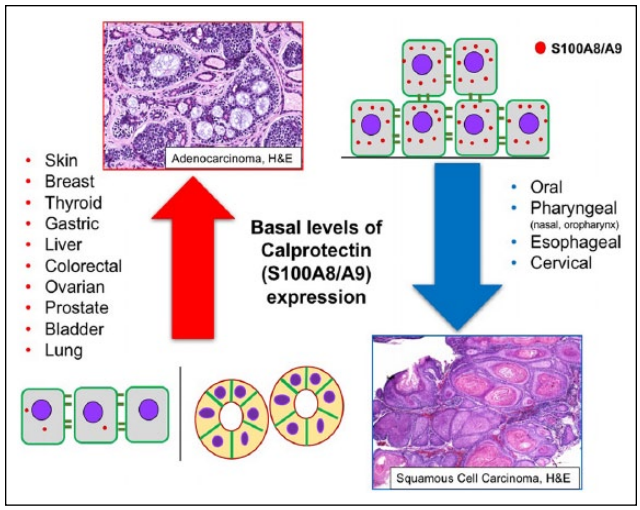

Figure 2. S100A8/A9 expression is differentially regulated in human malignancies. In tissues that express limited or no endogenous calprotectin (left side), such as the glandular breast epithelium or epidermis of the skin, calprotectin levels increase (red arrow) when tumorigenesis occurs; these neoplasms are generally diagnosed as adenocarcinomas. In tissues that constitutively express higher levels of calprotectin, including the oral mucosal epithelium (right side), squamous cell carcinomas show reduced calprotectin expression (blue arrow). Basal level of expression represents constitutive calprotectin expression in normal tissues. H&E, hematoxylin and eosin.

et al. 2003; Funk et al. 2015; Khammanivong et al. 2016). In oral, oropharyngeal, nasopharyngeal, esophageal (Kong et al. 2004; Wang et al. 2004), and cervical (Coleman and Stanley 1994; Tugizov et al. 2005) squamous cell carcinomas, calprotectin is significantly downregulated (Fig. 2, blue downward arrow). The differential control of calprotectin expression in normal epithelia and tissue-specific malignancies suggests epigenetic regulation during epithelial development and dysregulation associated with the initiation of certain cancers.

Structural Biology and Regulation of S100A8/A9

In humans, S100A8 and S100A9 typically form heterodimers (S100A8/A9); homodimers are not normally detectable but can form under certain conditions (Leukert et al. 2005). Monomers and homotrimeric or homotetrameric complexes are also possible (Nacken et al. 2003). As mentioned, S100A8/A9 expression is associated with early stages of myeloid cell differentiation and inflammation. Normally localized to the suprabasal spinous and keratin cell layers of stratified squamous mucosal epithelia (Hayashi et al. 2007), expression is most likely differentially regulated during each stage of cell differentiation.

The *S100A8* and *S100A9* genes encode no upstream signaling peptides, which are normally required to target proteins for export. Hence, the calprotectin complex is predicted to reside in the cytoplasm and is unlikely to be secreted by epithelium into extracellular space under normal conditions. Calprotectin has been reported to be released from aberrantly differentiated metaplastic primary human squamous tracheobronchial cells (Kim et al. 2007). In response to chronic exogenous stress, including inflammation, epithelial progenitor cells could therefore undergo metaplasia and release S100A8/A9. Some workers describe release as noncanonical secretion. Other than release during tissue and cell apoptosis or necrosis, secretion of extracellular calprotectin from normal epithelium has not been reported.

S100A8/A9, involucrin, and filaggrin genes are upregulated in human gingival keratinocytes in response to interleukin 1α (IL-1α) and calcium—2 factors known to promote keratinocyte differentiation (Hayashi et al. 2007). In contrast, transforming growth factor β (TGF-β), which inhibits proliferation and differentiation, downregulates calprotectin expression (Hayashi et al. 2007). Similarly, keratinocyte growth factor produced by mesenchymal cells appears to inhibit calprotectin expression (Bando et al. 2010), whereas the transcription factors C/EBPα and GLI, which regulate cellular growth and differentiation, appear to be essential for *S100A8* and *S100A9* expression (Cammenga et al. 2003; Tavor et al. 2003; Hayashi et al. 2007). In HaCaT keratinocytes, IL-1α is also suggested to induce *S100A9* expression by signaling through the IL-1 receptor and p38 MAPK, which increases the binding activity of C/EBPβ (Bando et al. 2013).

Regulation of calprotectin in epithelial cells may differ from other cell lineages. For example, in fibroblasts, transcriptional regulation of *S100A8* appears to be mediated by fibroblast growth factor 2 (FGF-2), IL-1β, and TGF-β (Rahimi et al. 2005), whereas *S100A9* expression in myeloid cells appears to be regulated by a myeloid-related regulatory element in the upstream promoter region; myeloid-related regulatory element is known to bind poly(ADP-ribose) polymerase 1 and the Ku70/Ku80 transcriptional complex (Kerkhoff et al. 2002; Grote et al. 2006). These several regulatory pathways could explain how calprotectin is differentially regulated depending on the cell lineage.

Calprotectin appears to be essential for development. Whereas not all cell types constitutively express calprotectin, mutation of the S100A8 subunit of the calprotectin complex causes rapid resorption of the mouse embryo by day 9.5 of development (Passey et al. 1999). Mutation of the S100A9 subunit also abrogates S100A8 protein production (but not gene expression), impairs myeloid cell function, and results in an S100A8/A9^{null} mutation (Hobbs et al. 2003; Manitz et al. 2003). Therefore, S100A8 appears to be critical for physiologic development and cellular function.

Role of Calprotectin in Head and Neck Carcinogenesis

Calprotectin and Oral and Oropharyngeal Squamous Cell Carcinoma

Head and neck cancer includes oral squamous cell carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC) and is the sixth-most prevalent cancer worldwide; incidence appears to be increasing in the last decade, primarily in

association with human papilloma virus (HPV) infection (El-Naggar et al. 2017). More than 90% of malignancies affecting the oral and oropharyngeal mucosae are SCCs (El-Naggar et al. 2017). In OSCC and OPSCC, *S100A8* and *S100A9* mRNAs (Roesch Ely et al. 2005; Sapkota et al. 2008; Khammanivong et al. 2016; Reckenbeil et al. 2016) and translated proteins (Funk et al. 2015) significantly decrease when compared with nonneoplastic stratified squamous oral and oropharyngeal epithelium. Approximately 90% of OSCC and OPSCC specimens show combined loss of *S100A8* and *S100A9* expression based on data from The Cancer Genome Atlas (TCGA; Khammanivong et al. 2016). Interestingly, *S100A8* and *S100A9* downregulation is similar across all tumor grades (T), independent of nodal involvement (N) or distant metastasis (M; Khammanivong et al. 2016), but is related to locoregional recurrence (Harris et al. 2015). Given that calprotectin expression and clinical staging (TNM) appear mutually independent, dysregulation of S100A8/A9 may be a feature of the initiation of oral and oropharyngeal tumorigenesis. Indeed, S100A8/A9 expression in oral premalignant (precancerous) epithelial dysplasia is lower than in otherwise healthy oral mucosal tissues, suggesting progressive loss of S100A8/A9 during oral carcinogenesis (Argyris et al. manuscript in preparation).

When expressed endogenously or ectopically, intracellular calprotectin appears to regulate the malignant characteristics of OSCC cells. Based on a well-differentiated human OSCC cell line (TR146 cells), cellular MMP-2 activity, cell invasion, and migration in vitro were increased when endogenous *S100A8* and *S100A9* expression was silenced via shRNA, as we reported (Silva et al. 2014). Conversely, when *S100A8* and *S100A9* were overexpressed with transfection of KB cells (S100A8/A9-negative HeLa-like carcinoma cell line), MMP-2 expression, invasiveness, and migratory potential were significantly inhibited (Silva et al. 2014). Hence, S100A8/A9 suppresses malignant features of carcinoma cells in vitro that are typically associated with tumor initiation and spread (Fig. 3).

When tested in vivo, calprotectin-negative KB and KB-EGFP cells formed significantly larger tumors than KB-S100A8/A9 cells in a mouse xenograft tumorigenesis model (Khammanivong et al. 2016). Indeed, in OSCC and OPSCC more globally, *S100A8* and *S100A9* coregulate with gene networks controlling cellular development and differentiation, cell-to-cell signaling and interaction, and cell morphology; S100A8/A9 appears to downregulate expression of genes associated with invasion and tumorigenesis (Khammanivong et al. 2016). Loss of S100A8/A9 in OSCC is associated with poor tumor differentiation (Argyris et al. manuscript in preparation) and increased DNA methylation (Khammanivong et al. 2016; Fig. 3).

Cytoplasmic calprotectin also functions in vitro to control the cell cycle DNA damage checkpoint at G2/M and impede proliferation (Khammanivong et al. 2013). Overexpression of S100A8/A9 enhances protein phosphatase 2A (PP2A) activity and activates phosphorylation of p-Chk1 at Ser345. PP2A appears to target and dephosphorylate mitotic p-Cdc25C at Thr48, allowing p-Chk1 (Ser345) to phosphorylate Cdc25C at the inhibitory residue, Ser216. Phosphorylated and inactivated

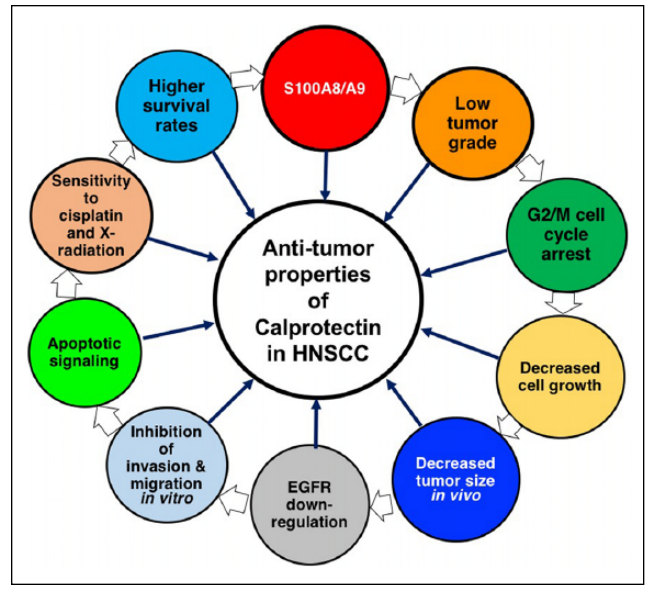

Figure 3. The multifaceted tumor-suppressive role of calprotectin in head and neck squamous cell carcinoma (HNSCC). When expressed in HNSCC, specifically oral and oropharyngeal, S100A8/A9 is associated with greater squamous differentiation (lower tumor grading) and activation of the G2/M DNA damage cell cycle checkpoint. These changes appear to cause attenuated cell growth in vitro and decreased tumor formation in vivo. In addition, epidermal growth factor receptor (EGFR), a negative prognostic factor, sustains proliferative signaling in the tumor milieu and is downregulated in S100A8/A9-expressing cell lines and tissues. Furthermore, calprotectin inhibits cancer cell migration and invasion, facilitates recruitment of DNA damage response proteins, and appears to promote apoptotic DNA fragmentation, thus conferring sensitivity to cisplatin and X-radiation. Finally, patients with S100A8/A9-high HNSCCs survive longer than patients with S100A8/A9-low or S100A8/A9-negative tumors. The white arrows do not necessarily indicate successive events but interconnected functions that collectively contribute (black arrows) to the antitumor properties of the calprotectin complex in HNSCC.

p-Cdc25C (Ser216) is targeted by 14-3-3β, which translocates both proteins for accumulation in the cytoplasm. As a consequence, the G2/M cyclin B1/p-Cdc2 (Thr14/Tyr15) complex remains inactive, arresting cell cycle at the G2/M DNA damage checkpoint (Khammanivong et al. 2013). Silencing of S100A8/A9 expression in the TR146 OSCC cells increased anchorage-dependent and anchorage-independent growth. S100A8/A9-mediated control of the G2/M DNA damage checkpoint is therefore a likely tumor-suppressive mechanism in human OSCC (Fig. 3).

Mutations of the tumor-suppressor *TP53* are detected in up to 72% of the HNSCC cases (Network 2015). Other frequently mutated genes include *CDKN2A, PIK3CA, NOTCH1, PTEN*, and *HRAS* (Network 2015; El-Naggar et al. 2017). Given that *S100A8* and *S100A9* are not frequently mutated in OSCC and OPSCC, epigenetic events may explain decreased S100A8/A9 expression in these neoplasms. Furthermore, epidermal growth factor receptor (EGFR) is highly upregulated in OSCC and OPSCC; EGFR expression is considered a negative prognostic indicator for disease progression and survival outcome (Mitsudomi and Yatabe 2010). In vitro and ex vivo experiments

showed that S100A8 and S100A9 levels were inversely correlated to membranous and cytoplasmic expression of EGFR (Argyris et al. manuscript in preparation). Given that calprotectin increases caspase 3/caspase 7 activity (Viemann et al. 2007; Ghavami et al. 2010) and that caspases 3 and 7 proteolytically cleave EGFR at the C-terminal region (Bae et al. 2001; Zhuang et al. 2003; He et al. 2006), S100A8/A9 may downregulate EGFR posttranslationally in OSCC and OPSCC through a caspase 3/caspase 7–dependent mechanism.

Consistent with a role for caspase activity causing downregulation of EGFR, S100A8/A9-high SCCs of the head and neck upregulate >363 apoptosis-related genes significantly more than S100A8/A9-low neoplasms, including *CASP1, -3, -4, -5, -7, -8, -9, -10*, and *-14*, based on analysis of data from TCGA. Furthermore, intracellular calprotectin appears to promote DNA fragmentation and cell death following radio- and chemotherapy, conferring S100A8/A9-expressing OSCC cells more sensitive to cisplatin and X-radiation (Argyris et al. manuscript in preparation). Notably, overall patient survival rates appear affected by calprotectin status; patients with S100A8- and/or S100A9-high HNSCC survive longer than patients with S100A8/A9-low tumors (Funk et al. 2015; Khammanivong et al. 2016; Fig. 3).

In S100A8/A9-high OSCCs and OPSCCs negative for HPV, calprotectin appears to function as a tumor suppressor, attenuating the malignant phenotype of carcinoma cells; our knowledge remains limited about the role of calprotectin in HPV-driven tumors. Calprotectin may inhibit viral oncogenic activity by regulating CKII-mediated E7 phosphorylation in vitro (Tugizov et al. 2005). Based on TCGA data, *S100A8* and *S100A9* expression tended to decrease more (nonsignificant) in HPV^+ than in HPV^- HNSCC samples (Khammanivong et al. 2016); the biological and prognostic significance of this observation is currently unknown. *S100A8* and *S100A9*, however, do not appear to be the only members of EDC regulated by HPV. Involucrin (*IVL*) and loricrin (*LOR*) are transcriptionally downregulated by E6 and E7 HPV oncoproteins in proliferating and differentiating human foreskin keratinocytes (Lehr and Brown 2003; Gyöngyösi et al. 2012). Specifically, involucrin is indirectly suppressed by E6 oncoprotein through HPV-mediated downregulation of transcription factor C/EBPα (Marthaler et al. 2017). C/EBPα, which regulates cellular growth and differentiation, appears to be essential for *S100A8* and *S100A9* expression (Cammenga et al. 2003; Tavor et al. 2003). HPV-driven decrease in C/EBPα may therefore also be responsible for S100A8/A9 downregulation in HPV^+ HNSCC.

Calprotectin and Esophageal Squamous Cell Carcinoma

Esophageal squamous cell carcinoma (ESCC) represents the eighth-most common type of human cancer and is characterized by poor prognosis; 5-y survival is 19% for all ESCC cases and only 0.9% for advanced tumors (Testa et al. 2017). The role of S100A8 and S100A9 in the initiation and progression of esophageal carcinogenesis has been described in in vitro and in vivo studies. Epidemiologic studies of ESCC suggest that zinc deprivation may be a major etiologic factor (Yang 1980; van Rensburg 1981). With special diets, zinc-deprived hyperplastic and zinc-replenished rat esophageal tissues were modeled (Taccioli et al. 2009). The hyperplastic zinc-deficient esophagi showed a distinct gene expression signature with 57- and 5-fold greater expression of *S100A8* and *S100A9* mRNA levels, respectively, than the zinc-replenished tissues (Taccioli et al. 2009). Nutritional replenishment of zinc levels restored S100A8 and S100A9 gene expression and the physiologic esophageal phenotype in vivo.

ESCC from 4-nitroquinoline 1-oxide (4-NQO)–treated, zinc-deficient $p53^{+/-}$ mice demonstrated increased S100A8 immunoreactivity, while nonneoplastic control zinc-sufficient $p53^{+/-}$ esophagi displayed weak S100A8 expression. Prolonged zinc deficiency in rats (21 wk) combined with noncarcinogenic low doses of N-nitrosamethylbenzylamine elicited a 66.7% incidence of ESCC (Taccioli et al. 2012). Dysplastic (precancerous) and neoplastic zinc-deficient esophagi showed 2-fold greater upregulation of *S100A8* and *S100A9* than the zinc-sufficient rat esophagi (Taccioli et al. 2012). When 4-NQO was applied topically to induce oral-esophageal tumorigenesis in zinc-deficient $COX\text{-}2^{-/-}$ mice, both *S100A8* and *S100A9* were upregulated in the precancerous forestomach (Wan et al. 2011). In these mice, the RAGE-S100A8/A9 inflammatory axis appeared activated in premalignant and carcinomatous lesions of the forestomach and tongue (Wan et al. 2011). Hence, zinc deficiency in this model appears to regulate S100A8 and S100A9 expression and modulates the link between S100A8/A9-RAGE interaction and downstream NF-κB/COX-2 signaling, driving esophageal cell proliferation and carcinogenesis. How zinc chelation by calprotectin (Clark et al. 2016) might contribute is unknown. Interestingly, signaling through the S100A8/A9-TLR4 pathway could contribute to the early development of esophageal cancer in a RAGE-independent manner, since $RAGE^{-/-}$ mice showed increased Toll-like receptor 4 (TLR4) mRNA and protein levels (Mark et al. 2013).

In human ESCC tissues ex vivo, S100A8 was detected in all specimens (n = 16); adjacent nonneoplastic stratified squamous esophageal epithelium showed negative or low S100A8 immunoreactivity (Taccioli et al. 2009). S100A9 protein was expressed in 89.5% of human ESCC samples (n = 57) and only 66.7% of nonneoplastic epithelium according to immunohistochemistry (Fan, Gao, et al. 2012). Whereas S100A8 and S100A9 were reported to be upregulated in human ESCC (Taccioli et al. 2009; Fan, Gao, et al. 2012), 11 of 16 S100 genes, including *S100A8* and *S100A9*, have been reported to be downregulated in 62 ESCC cases when compared with adjacent nonneoplastic esophageal epithelium (Ji et al. 2004). Specifically, *S100A8* and *S100A9* expression was downregulated in 82.3% (51 of 62) and 77.4% (48 of 62) of ESCC, respectively (Ji et al. 2004). Similarly, *S100A8, S100A9*, and cytokeratins (*KRT4* and *KRT13*), genes implicated in squamous cell differentiation and maturation, were coordinately downregulated in ESCC (n = 5) in comparison with adjacent nonneoplastic esophageal mucosal tissues, according to cDNA microarray and Northern blot analysis (Luo et al. 2004). Based on immunohistochemistry, S100A8 and S100A9 were decreased in 87% and 84% (n = 31) of human ESCC tumors, respectively;

increased S100A8/A9 expression levels were associated with greater differentiation (Luo et al. 2004). In a Chinese cohort (n = 64), S100A9 expression in ESCCs decreased in 91% of the specimens (Wang et al. 2004). This ex vivo data underscores the lack of consensus about the role of S100A8 and S100A9 proteins in human esophageal carcinogenesis.

Calprotectin and Nasopharyngeal and Laryngeal Squamous Cell Carcinoma

The role of S100A8/A9 in nasopharyngeal, pharyngeal, and laryngeal cancer is underinvestigated. In nasopharyngeal squamous cell carcinoma (NSCC), S100A8 and S100A9 expression was suppressed in vitro in 7 carcinoma cell lines as evidenced by cDNA array hybridization and reverse transcription polymerase chain reaction analysis (Fung et al. 2000). S100A8 and S100A9 protein expression was apparently absent in pharyngeal epithelial malignancies but expressed in the superficial layer of nonneoplastic pharyngeal epithelium according to ProteinChip arrays (Melle et al. 2004). In contrast, S100A9 protein levels appeared significantly greater in the stroma of NSCC cases (n = 66) than normal nasopharyngeal epithelial tissue (Li et al. 2009). S100A9 immunoreactivity varied in stromal inflammatory cells in all NSCC cases, whereas the carcinoma cells and normal pharyngeal epithelial cells were uniformly negative for S100A9 (Li et al. 2009). In more advanced NSCC with increased regional lymph node metastasis, S100A9 was upregulated in the inflamed tumor microenvironment but not in the carcinoma cells per se (Li et al. 2009).

In laryngeal squamous cell carcinoma (LSCC) specimens (n = 2), S100A9 protein levels were lower than in adjacent nonneoplastic tissues as assayed with 2-dimensional gel electrophoresis and mass spectroscopy (Sewell et al. 2007). In Hep-2 LSCC cells, the 3′UTR of S100A8 harbors a specific binding site for miR-24 in-vitro (Guo et al. 2012). Whereas ectopic expression of miR-24 had no significant effect on S100A8 mRNA levels, S100A8 protein significantly decreased when Hep-2 cells were transfected with miR-24 (Guo et al. 2012). miR-24 therefore appeared to negatively regulate S100A8 expression via translational repression. In contrast to OSCC and OPSCC, S100A8 protein may be associated with tumor invasion in LSCC. After S100 antibody blockade, miR-24 significantly inhibited invasion of Hep2 cells in vitro (Guo et al. 2012). Hence, the role of S100A8, S100A9, and calprotectin complex in LSCC requires further investigation.

Calprotectin and Thyroid Adenocarcinoma

In thyroid cancer, S100A8/A9 does not appear to play a significant role except for an anaplastic and clinically aggressive subtype, although the literature is sparse. Nonneoplastic follicular cells of the thyroid gland do not express S100A8 or S100A9 proteins, and both proteins are absent in normal thyroid tissue and benign neoplasms, including follicular adenoma (Ito et al. 2005; Ito et al. 2009). Thyroid malignancies, including follicular and papillary carcinomas with conventional tumor architecture, are also uniformly S100A8 and S100A9 negative. A few follicular and papillary thyroid carcinomas with atypical growth patterns show S100A9 immunoreactivity in <5% of neoplastic cells (Ito et al. 2005; Ito et al. 2009). In contrast, thyroid adenocarcinomas with high-grade transformation (dedifferentiated, anaplastic), poorer prognosis, and more aggressive tumor behavior showed significantly increased S100A8/A9 immunohistochemical positivity (Ito et al. 2005; Ito et al. 2009). Elevated calprotectin protein levels, therefore, may drive dedifferentiation and high-grade transformation in thyroid cancer.

In anaplastic thyroid carcinoma (ATC), S100A8/A9 mRNA and protein levels are greater than in nonneoplastic thyroid tissue and well-differentiated thyroid tumors (Reeb et al. 2015). ATC cell lines proliferate in response to exogenous S100A8 in vitro (Reeb et al. 2015). Knockdown of endogenous S100A8 through shRNA techniques inhibited S100A8-mediated ATC cell proliferation in vitro and tumor formation in vivo; animal survival improved (Reeb et al. 2015). S100A8 appears to stimulate cell growth by interacting with RAGE and activating 3 downstream MAPK pathways: p38, ERK1/2, and JNK (Reeb et al. 2015). Interestingly, the oncogenic and metastatic potential of ATC cells was independent of the status of S100A9. Hence, therapeutic targeting of S100A8 may prove beneficial for patients with ATC.

In patients with papillary thyroid carcinoma, the oxidative stress index, serum lipid hydroperoxides, and calprotectin levels correlate and appear to increase (Tabur et al. 2015). After total thyroidectomy, the levels of serum S100A8/A9 substantially decrease (Tabur et al. 2015). Hence, calprotectin may be a useful biomarker for this type of thyroid malignancy.

Conclusion

In contrast to malignant neoplasms of other anatomic sites, intracellular S100A8A/A9 appears to play a multifaceted tumor-suppressive role in HNSCC, specifically oral and oropharyngeal tumors, by regulating tumor differentiation, restoring the G2/M cell cycle checkpoint, and inhibiting invasion and migration in vitro and tumor formation in vivo. Conversely, loss of calprotectin expression associates with upregulation of the negative prognosticator EGFR, resistance of carcinoma cells to chemotherapeutics and X-irradiation, and poorer patient survival rates. Notably, decrease or loss of calprotectin at the stage of preneoplasia by a subset of dysplastic cells may accelerate cell proliferation and induce expression of cancer-promoting genes, therefore contributing to transformation toward HNSCC. The reviewed anticancer properties of calprotectin in HNSCC (Fig. 3) suggest targets for new therapeutic strategies. Indeed, calprotectin itself may represent a therapeutic target. We speculate that restoration of endogenous levels of S100A8/A9 in dysplastic cells of oral premalignant epithelial lesions or existing tumors could prevent or decelerate malignant transformation

and invasion of the basement membrane, reduce cell proliferation via arrest of the cell cycle at G2, indirectly promote EGFR cleavage, augment the DNA damage repair response, and increase the efficacy of established therapeutic regimens, including radio- and chemotherapy.

Author Contributions

P.P. Argyris, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Z.M. Slama, contributed to data acquisition and analysis, critically revised the manuscript; K.F. Ross, M.C. Herzberg, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; A. Khammanivong, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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