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ORIGINAL ARTICLE

Lack of modulatory function of coding nucleotide polymorphism S100A2_185G>A in oral squamous cell carcinoma

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OBJECTIVE: S100A2, a Ca^{2+} -binding protein with two EF-hands, is a tumor suppressor in oral cancer. Helix III flanking the C-terminal EF-hand is implicated to participate in the interaction of S100A2 and its target(s). The aim of this study was to examine if the coding sequence polymorphism S100A2_185G>A, leading to the peptide 62 substitution of asparagine (AAC, A allele) for serine (AGC, G allele) in helix III, had modulation effects on S100A-mediated tumor suppression.

SUBJECTS AND METHODS: We sequenced the coding sequence of S100A2 gene in normal oral keratinocytes (NOKs), dysplastic oral keratinocytes (DOKs), eight oral cancer lines, and 54 pairwise oral cancer specimens. We also compared the *in vitro* anti-tumor effect of wildtype (G allele) and variant (A allele) S100A2 expression using cell proliferation, migration, invasion, and colony formation assays.

RESULTS: With the exception of CAL27 and SCC-15 cancer lines being heterozygotes of A and G alleles, the remaining oral cells were homozygotic in G alleles. No alterations of anti-growth, anti-migration, anti-invasion, and anti-colony formation were observed between variant and wildtype cells. Moreover, no minor S100A2_185A allele was detected in 54-pairwise clinical specimens.

CONCLUSION: The coding sequence polymorphism S100A2_185G>A had no regulatory role in S100A2-mediated tumor suppression in oral cancer.
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Keywords: S100A2; polymorphism; tumor suppressor; oral cancer

Introduction

S100 family constitutes the largest subfamily of the EF-hand proteins. S100 protein binds to Ca^{2+} via EF-hands. Each member contains two EF-hands connected by a central hinge, being S100 family-specific in the N-terminus and canonical in C-terminus. The N-terminal EF-hand consists of 14 amino acids flanked by helix I and II whereas the C-terminal one has 12 amino acids flanked by helix III and IV (Fritz and Heismann, 2004). There are at least 25 members in human genome. Twenty-one of them cluster in chromosome 1q21 (Santamaria-Kisiel *et al*, 2006). This region also encodes many other genes that are highly expressed in epidermal keratinocytes and is called as epidermal differentiation complex (Marenholz *et al*, 1996; Li *et al*, 2000). The location of most S100 genes in this region has heightened interest in their role in epidermal differentiation.

Many S100 genes are found associated with various human diseases like psoriasis (Heizmann, 2002; Eckert *et al*, 2004). S100A2 is a member believed to be a tumor suppressor due to its frequent down-regulation in several cancer types. We and others have shown that the expression of S100A2 not only serves as a prognostic marker in several cancer types (Maelandsmo *et al*, 1997; Kyriazanos *et al*, 2002; Suzuki *et al*, 2005; Tsai *et al*, 2005) but also plays a role in suppressing

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metastasis (Zhang *et al*, 2007). Consistent with the notion, ectopic overexpression of S100A2 in little-S100A2 expressing carcinoma cells attenuates the ability of cancer cells to grow, migrate, invade Matrigel, and form colonies in soft agar and tumors in nude mice (Tsai *et al*, 2006). Knocking down the expression of S100A2 restrained head and neck cancer cell migration (Nagy, 2001).

Four nucleotide polymorphisms leading to amino acid substitutions have been identified in the coding region of S100A2 gene. One of them resides in the peptide residue 62. This coding sequence variation existing in the major G allele (94.9%) and the minor A allele (5.1%) was previously reported in the study of psoriatic patients (Stoll *et al*, 2001). This allelic change from G to A in coding nucleotide 185 results in the peptide 62 substitution of asparagine for serine (S62N). S100A2 protein contains four alpha-helixes involving residues 7–21 (I), 31–40 (II), 56–64 (III), and 72–95 (IV). Residue 62 is the seventh peptide in helix III, upstream from canonical EF-hand (Fritz and Heismann, 2004). Upon Ca^{2+} binding, S100 protein undergoes a conformational change followed by the exposure of a hydrophobic cleft composed of the central hinge, helix III, and C-terminal loop region. The accessible hydrophobic residues in the cleft would allow the binding of target proteins to S100A2 (Marenholz *et al*, 2004). Thus S62N in the cleft region might have impacts on calcium binding-induced association of S100A2 with its targets, consequently the anti-tumor effect mediated by S100A2.

A recent study in lung cancer showed that S62N polymorphism was significantly found in 14.4% of all tested non-small cell lung carcinoma samples, suggesting a functional role of this polymorphism (Strazisar *et al*, 2009). However, as no direct evidence to address whether this coding sequence 185G > A polymorphism has any effect on S100A2-mediated functions; we used site-directed mutagenesis to create A allele (S100A2_185A) in carcinoma cells and verify its consequences in vitro. The genotypes of S100A2 in this nucleotide in 54 pairs of normal and oral squamous cell carcinoma (SCC) specimens were also examined.

Materials and methods

Materials

Fetal bovine serum (FBS) was from Biological Industries, Israel. Human keratinoctye growth supplement for growing normal oral keratinocytes (NOKs) was from Cascade Biologics, Inc. (Portland, OR, USA). All the other culture medium and antibiotics were from Gibco (Carlsbad, CA, USA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). G418 and oligonucleotide primers were from MDbio, Inc. (Taipei, Taiwan). A mouse monoclonal antibody against S100A2 (clone 14) was from BD Biosciences (San Jose, CA, USA). Anti-β-actin antibody was from Chemicon (Billerica, MA, USA). Chemiluminescence Reagent Plus was from NEN Life Science Products (Boston, MA, USA). Phenol-red free Matrigel was from BD Biosciences (Bedford, MA, USA). All PURGENE kits were from Gentra Systems (Minneapolis, MN, USA). Miscellaneous reagents for RT-PCR and cloning were from Promega (Madison, WI, USA).

PCR and sequencing analysis of the protein coding regions of S100A2 gene

S100A2 protein coding sequence resides only in the second and third exons. The third exon contains the polymorphic allele (185G > A). Primers for exon 2 and 3 are as follows: S100A2-E2-F: 5′-GGCATAGGGCAGAAGTGGA-3′; S100A2-E2-R: 5′-ACAGATGGACTCGGAAGGC-3′; S100A2-E3-F: 5′-GCTGGAACCAACCTGAATG-3′; S100A2-E3-R: 5′-GACCTAAAGCATAGCTCTGGC-3′. Total genomic DNA for cultured cells was prepared by PUREGENE DNA purification kits. PCRs were performed with the primers for exons 2 and 3 and 300 ng of genomic DNA. The PCR condition was 35 cycles of 94°C (15 s), 60°C (45 s), and 72°C (20 s). PCR products were purified using PCR purification kit (Geneaid, Taipei, Taiwan) prior to automated DNA sequence analysis.

Maintenance of cultured cells

Normal oral keratinocytes were isolated and maintained as described (Tsai *et al*, 2005). Dysplastic oral keratinocytes (DOKs) and eight oral cancer lines, CAL-27, SCC-9, SCC-15, SCC-25, OC-2, OC-3, OEC-M1, and HSC-3 were maintained as described (Chang *et al*, 1992; Matsui *et al*, 1998; Lin *et al*, 2004; Tsai *et al*, 2005). KB carcinoma cells with little S100A2 expression were maintained as described by American Tissue Type Collection.

Patient samples and genomic DNA isolation

This study included 54 Han Chinese patients with histopathologically defined SCC in their oral cavity. Oral cancer specimens, blood, or buccal cell collection were approved by the Institutional Review Boards and obtained with informed consent from these patients undergoing surgery at National Cheng Kung University or Chang Gung Memorial Hospitals. We individually twirled sterile cytology brushes (Medical Packaging Corp., Camarillo, CA, USA) on tumor and normal parts in the inner cheek of each patient for 30 s to collect buccal cells. We then used PUREGENE Buccal Cell Kits to isolate buccal genome on the brushes and PUREGENE DNA purification kits to isolate blood genome.

Site-directed mutagenesis

The G185A-S100A2 expression construct was generated by QuickChange Site-Directed mutagenesis kits (Stratagene, La Jolla, CA, USA) using wildtype S100A2-bearing pcDNA3.1 vector as the template. The complementary primer pairs for mutagenesis are: forward, 5′-GAAGCTGATGGGCAACCTGGATGAGAACAG-3′; reverse, 5′-CTGTTCTCATCCAGGTTGCCCATCAGCTTC-3′. The underlined, mutated sequence was verified by automated DNA sequencing.

Transfection and establishment of stable clones
Human carcinoma KB cells lack of detectable S100A2 protein expression were seeded overnight into 35-mm culture dishes in appropriate growth medium supplemented with 10% FBS before transfection with 1 μg wild-type, G185A-S100A2-expressing vector, or empty expression vector using Lipofectamine 2000. Twenty-four hours post-transfection, stable vector or S100A2-expressing clones (S100A2-WT *vs* S100A2-G185A) were established using 800 μg ml^{-1} G418 and expanded into individual cell clones.

Western blot analysis
Protein lysates for stable clones were harvested using boiled lysis buffer containing 1% SDS and 10 mM and Tris–HCl (pH 7.4). Equal amounts of total protein from each sample were fractionated by SDS–PAGE and blotted onto a PVDF membrane, and probed with an anti-human S100A2 antibody and then a secondary antibody. The hybridized immunocomplex was detected by Renaissance Chemiluminescence Reagent Plus.

Cell proliferation assay
Cells from vector, WT-1, WT-2, G185A-1, or G185A-2 clones were plated at a density of 3000 cells per well in quadruplicates in a 96-well tissue culture plate. After 24, 48, and 72 h, cell proliferation was measured using Cell Titer 96 One Aqueous Cell Proliferation kit (Promega, Madison, WI, USA). The same experiment was independently repeated three times and results were expressed as mean ± s.d.

Migration and invasion assays
Migration and invasion assays were performed in 24-well Transwell units with 8-μm-pore polycarbonate membranes (Corning Costar, Cambridge, MA, USA). For migration, cells (1×10^5 per well) in 100 μl of the growth medium were added to the upper chamber and fed with 500 μl growth medium in the lower chamber. After 8-h incubation, cells that remained on the upper side of the filter were removed with cotton swabs. For invasion, cells at the seeding density were added to the upper chambers coated with 1 mg ml^{-1} Matrigel and fed with 500 μl growth medium in the lower chamber. Following 24-h incubation, cells in the upper chamber were removed with cotton swabs. Migratory or invasive cells on the lower surface were stained with Giemsa solution and counted in five random fields under a light microscope at 400 × magnification. Each experiment was performed in duplicate wells and repeated three times, and results were expressed as mean ± s.d.

Adhesion assay
Cells (1×10^5) from each clone were seeded on Matrigel (1 mg ml^{-1})-coated 96-well microtiter plates. After 60-min incubation at 37°C in a 5% CO_2-incubator, the plates were rinsed twice with phosphate-based buffer (PBS) and shaken vigorously on a plate shaker for 15 s to remove non-adherent cells. Adherent cells were stained by 1% crystal violet for 1 h. The purple color was extracted by 2% SDS and quantified by spectrometer.

Soft agar assay
Briefly, 1×10^4 cells of each stable cell clone at single-cell suspension were seeded in duplicate onto 60-mm culture dishes in MEM containing 0.35% agarose overlying a solidified 0.7% agarose under layer. After the cell-containing layer solidified, 0.7% agarose was overlaid. Cells were fed every 4 days and incubated at 37°C in 5% CO_2 for 5 weeks. The colony number and diameter was measured by ScopePhoto software (Micro Imaging, Ltd., Auckland, New Zealand). Twenty low power fields (40 × magnifications) of each clone were calculated. Each experiment was performed in duplicate and repeated three times, and results were expressed as mean ± s.d.

Statistical analysis
Statistical significance was tested by Student's *t*-test for either paired or unpaired data as appropriate.

S100A2 protein structure modeling
The S100A2 protein crystal structure was retrieved from protein data bank (code: 2RGI). The S62N mutation was modified and the electro-potential map was calculated by 2008 Molecular Operating Environment version 10 (Chemical Computing Group, Inc., Montreal, Canada).

Results

Sequence analysis of S100A2 coding sequence
The protein coding sequence of S100A2 is encoded by exons 2 and 3. We sequenced both exons 2 and 3 in NOKs, precancer DOKs and eight oral cancer cell lines. Among the oral cells we tested, NOKs, DOKs, OC-2, OC-3, OEC-M1, SCC-9, and SCC-25 were homozygotic G alleles at nucleotide position 185 without any other variations. The remaining two cell lines, CAL-27 and SCC-15, harbored two nucleotide variations in exon 3 but not exon 2. Both cells were heterozygotes of G and A alleles in the same position (Table 1). We also found the heterozygosity of another non-coding sequence variation, S100A2_T314C, locating at 17 bases downstream from the last nucleotide of stop codon in these two lines (Table 1). Together, both coding (G185A) and non-coding sequence (T314C) variations in the exon 3 were only detected in CAL-27 and SCC-15 cells.

No effects of 185G > A or 314T > C polymorphisms on S100A2 mRNA and protein expression
As coding or non-coding polymorphisms might have impacts in mRNA or protein stability, we examined if these variations had any effect on the expression of S100A2 mRNA or protein. Semi-quantitative RT-PCR and Western blot analyses were used to measure S100A2 mRNA and protein expression in NOKs with common G/G genotype, four oral lines with G/G or G/A genotypes, and KB carcinoma cells with A/A genotypes in this nucleotide. KB cells served as a control for minor S100A2_185A allele. Regardless of the genotypes in the nucleotide 185 position, the expression of S100A2 mRNA was differentially reduced in these cells when compared with NOKs (Figure 1a). Despite of the

Table 1 Polymorphism in the exon 3 of S100A2 gene in NOK and nine oral cell lines

Cell line	*Nucleotide 185 (CDS)*	*Amino acid sequence*	*Nucleotide 314 (3′UTR)*
NOK	G/G		
DOK	G/G		
OC-2	G/G		
OC-3	G/G		
OEC-M1	G/G		
CAL-27	G/A	$62^{Ser/Asn}$	T/C
SCC-9	G/G		
SCC-15	G/A	$62^{Ser/Asn}$	T/C
SCC-25	G/G		
HSC-3	G/G		

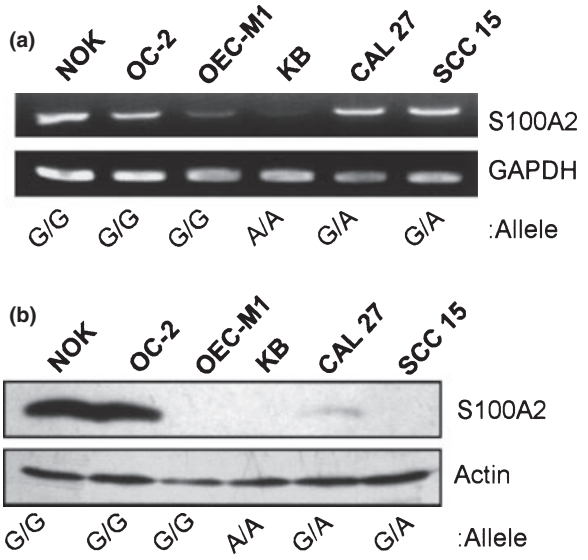

Figure 1 Effect of 185G > A polymorphism on S100A2 mRNA and protein expression. (**a**) Semi-quantitative RT-PCR analysis of S100A2 mRNA in NOK, KB and four oral cell lines with the indicated genotypes at the bottom. GAPDH, a loading control. (**b**) Western blot analysis of NOK, KB and four oral cancer lines. Actin, a loading control

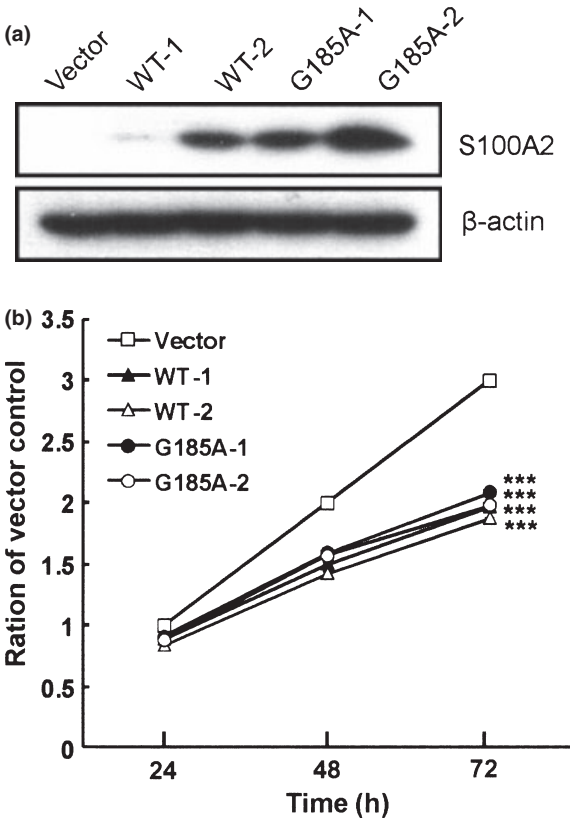

Figure 2 Ectopic wildtype or G185A variant of S100A2 significantly attenuated carcinoma cell growth. (**a**) Western blot analysis of S100A2 protein in wildtype and G185A-S100A2 stable clones. WT-2 clone expressed a higher level of S100A2 protein than WT-1 whereas G185A-2 had a higher expression of S100A2 protein than that G185A-1. β-actin, a loading control. (**b**) Cell proliferation assays of the indicated cell clones at 24-, 48-, and 72-h postseeding. Regardless of the type or amount of S100A2 protein in the cells, the presence of S100A2 protein significantly slowed down cell growth when compared with vector control cells. $^{***}P < 0.001$ *vs* vector control. Each experiment was performed in quadruplicate and repeated three times, and the results were expressed as mean ± s.d

presence of S100A2 mRNA in OEC-M1 (G/G), KB (A/A), CAL27 (G/A), and SCC15 (G/A), little or no S100A2 protein was detected in these cells, suggesting a post-transcriptional regulation of S100A2 expression in these cells (Figure 1b). OC-2 cells are the only cells with concordant expression of both S100A2 mRNA and protein. Together, there was no obvious correlation between S100A2 genotypes in this locus and the relative S100A2 mRNA or protein expression in these cells.

Establishment of stable clones expressing differential levels of WT-S100A2 or G185A-S100A2

As this minor S100A2_185A allele leads to amino acid substitution at residue 62, we used site-directed mutagenesis to generate the A allele-encoding expression vector for the study of allelic change impacts on the antitumor effect of S100A2. KB carcinoma cells, with A/A genotype in this nucleotide and residual S100A2 protein, were used as host cells for establishing S100A2_185G-expressing wildtype clones (WT-1 and WT-2), S100A2_185A variant clones (G185A-1 and G185A-2), and a pool of vector control clones. Western blot analysis confirmed the differential levels of S100A2 and its variant proteins in these clones (Figure 2a). The level of wildtype S100A2 protein was higher in clone 2 (WT-2) than that in clone 1 (WT-1). Likewise, the expression of S100A2 variant was also higher in clone 2 (G185A-2) than clone 1 (G185A-1). S100A2 protein was not detected in vector control cells. These clones were used for the following assays.

Reduction of cell growth by wildtype S100A2 or its minor variant

Ectopic S100A2 expression reduced cell growth (Tsai *et al*, 2006). Growth curve assay was used to unravel if a single amino acid substitution at codon 62 in S100A2 had any effect on cell growth. The cells expressing wildtype S100A2 or variant S100A2-G185A protein had significantly slower growth rate than those expressing vector control (Figure 2b). Although WT-2 clone

expressed a similar level of wildtype S100A2 to that of variant S100A2 in G185A-1 clone (Figure 2a), there was no difference in their growth ability. Moreover, WT-1 cells, with a lower level of wildtype S100A2 than WT-2 cells, had similar growth rate to WT-2 cells. Regardless of the genotype in the 185 nucleotide position and the expression level of S100A2, ectopic S100A2 expression exerted anti-proliferation effect.

Inhibition of cell migration, invasion and colony formation by wildtype S100A2 or its variant

S100A2 had anti-migration, anti-invasive and anti-colony forming abilities (Tsai *et al*, 2006). We first examined if the codon 62 variation had effects on the ability of KB cells to migrate or invade Matrigel. Stable clones expressing wildtype S100A2 (WT-1 and -2) or minor variant S100A2_185A allele (G185A-1 and -2) were used for migration or invasion assays. The migration and invasive ability of cancer cells expressing either wildtype (WT-1 and -2) or variant S100A2 (G185A-1 and -2) was dramatically reduced when compared with vector control cells ($P < 0.001$) (Figure 3a,b). However, WT-2 clone tended to migrate or invade through Matrigel slower than G185A-1 clone although both expressed similar levels of wildtype or variant S100A2. To confirm the decrease of cell migration and invasion was not due to the S100A2-meidated inhibition of cell adhesion, we performed cell adhesion assay. No difference in the adhering ability of vector control and S100A2-expressing cells were detected (Figure 3c). Although more S100A2 variant protein was required to inhibit cell migration than its wildtype counterpart, both S100A2 and its variant manifested a potent inhibitory effect on cell migration and invasion.

We then measured the effect of ectopic S100A2 on anchorage-independent growth using soft agar assays. After 5 weeks, the number of colony size larger than 100 μm was significantly reduced by both forms of S100A2 protein (Figure 3d). Although S100A2-expressing KB cells still formed colonies in soft agar, the size of colonies was decreased. No S100A2-expressing colonies larger than 100 μm was detected. These data indicate that both wildtype and variant S100A2 suppressed the anchorage-independent growth of carcinoma cells. Taken together, the amino acid substitution of asparagine for serine on residue 62 had no obvious impact on the anti-migration, anti-invasion or anti-anchorage independence mediated by S100A2.

No obvious structure-wise change induced by the substitution of asparagine for serine at residue 62 in S100A2

Other than a minor loss of anti-migration effect exerted by S100A2_185A allele, there was no obvious impact of this sequence variant in the ability of S100A2-excpressing cells to proliferate, migrate, invade Matrigel or form colonies on semi-solid agar. Since the crystal structure of S100A2 has been published (Koch *et al*, 2006), we used molecular modeling software to compare the S100A2 protein structure before and after the amino acid substitution at residue 62. Consistent with our various in vitro assays, we could not find any obvious structure-

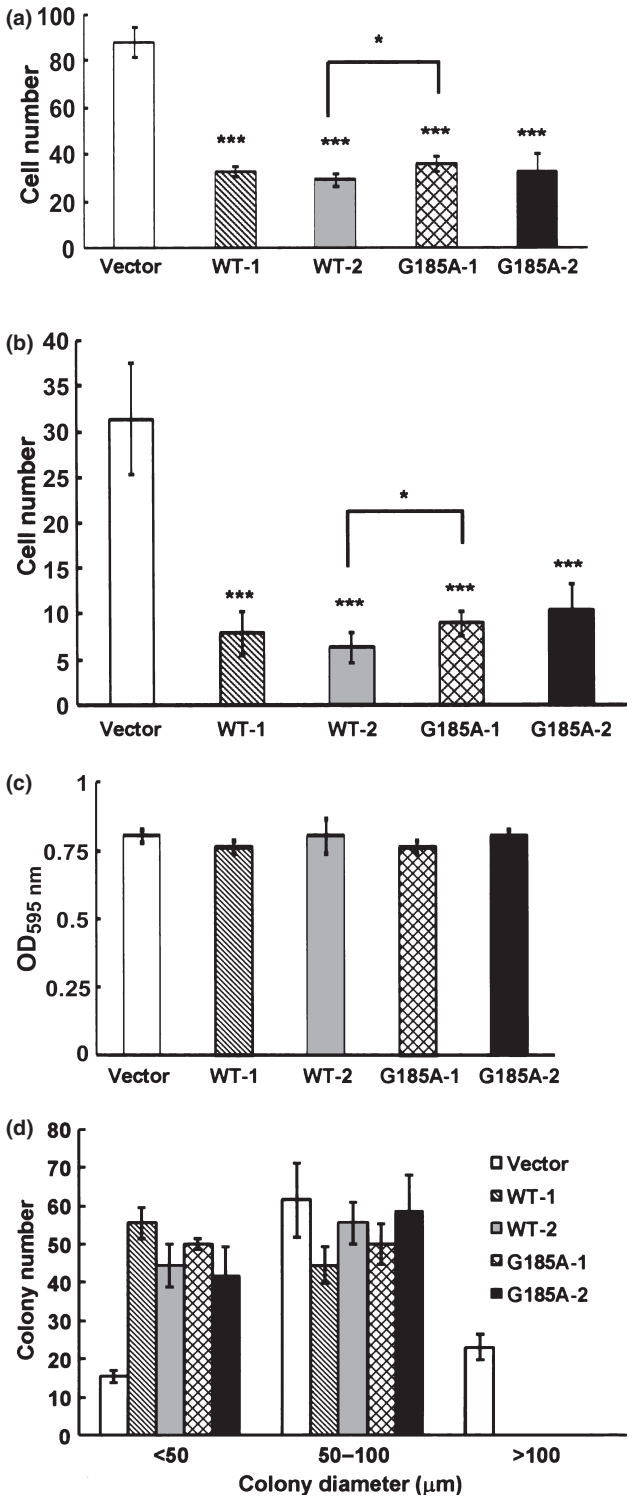

Figure 3 Ectopic wild-type or S100A2 variant significantly attenuated cell migration, invasion and anchorage-independent growth. We performed (**a**) migration, (**b**) invasion, (**c**) adhesion, and (**d**) colony formation assays to measure the ability of vector, WT-1, WT-2, G185A-1, and G185A-2 clones to migrate, invade Matrigel, adhere to Matrigel-coated dishes, and form colonies on semisolid agar. Colony size in soft agar assay was calculated by ScopePhoto software and divided into three groups (<50, 50–100, and >100 μm). Each experiment was performed in duplicate and repeated three times, and the results were expressed as mean ± s.d. $^{*}P < 0.05$ *vs* WT-2. $^{***}P < 0.001$ *vs* vector control

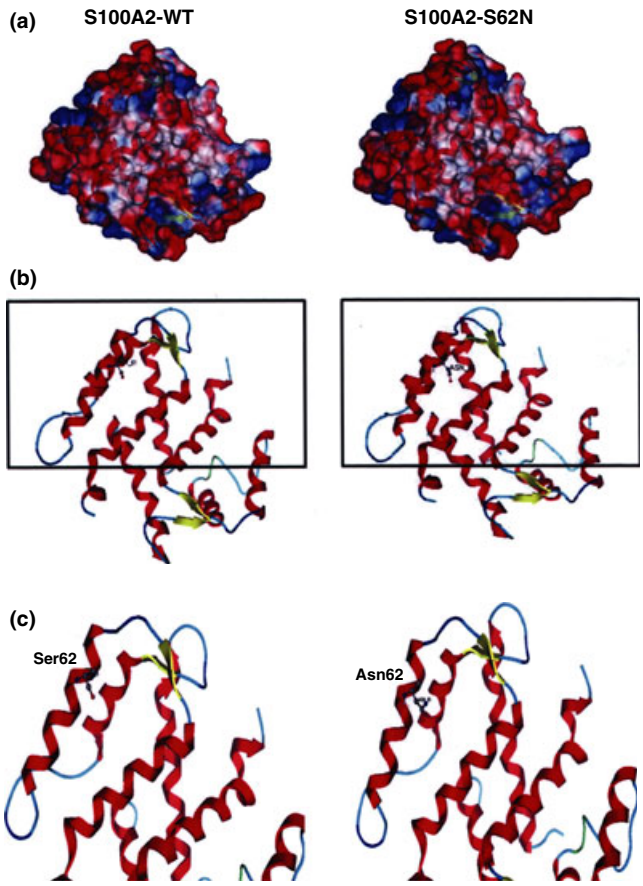

Figure 4 Structure comparison between wild-type and S62N-S100A2 variant. (**a**) Electrostatic potential surface representation of the wild-type S100A2 (PDB entry 2RGI, left) and S62N-S100A2 (right). Partial charges were calculated with Amber 99 force field and projected on the protein surface, whereas colored patches (red = negative, blue = positive, white = neutral) via Molecular Operating Environment (ver. 2008 10). There are no major differences of the surface potential between the two structures. (**b**) Stereo view of the protein structure (depicted as helical model) points out the related locations of the ser62 and Asn62 (depicted as stick-and-ball models), respectively. (**c**) A closer view of residue 62

wise change of S100A2 protein (Figure 4a–c), indicating a neutral function of asparagine substitution for serine at residue 62.

No tumor-specific coding variation of S100A2 at residue 62 in the clinical specimens

We further extrapolated our in vitro findings to *in vivo*. Fifty-four oral cancer patients, including 52 males and two female with a median age of 52, were recruited with informed consent for this study. Their clinicopathological characteristics are listed in Table 2. Adjacent normal oral mucosa or blood from the same patient was used as normal control. After DNA sequence analysis of the exon 3 region in 108 chromosomes from 54 pairwise patient samples (tumor *vs* normal), no A allele carrier manifested as G/A heterozygotes and A/A homozygotes was identified in these patients. Together, no minor S100A2_185A allele was detected in any of the 54 pairwise oral SCC tumor and their adjacent normal tissues.

Table 2 Clinicopathological characteristics of 54 oral cancer patients

Age (n = 54)	*37–77 (median: 52)*
Gender	52 males and 2 females
Smoking	45 (83%)
Alcohol drinking	43 (80%)
Betel quid chewing	40 (74%)
Differentiation	
Well	37
Non-well	17
Tumor sites	
Buccal	24
Tongue	12
Others	18
Staging	
I	16
II	12
III	8
IV	18

Discussion

We have shown that minor A allele behaved similarly to common G allele in the reduction of cell growth, migration, invasion into Matrigel and colony formation in soft agar. Although this amino acid substitution (S62N) resides in the region responsible for target protein binding, no attenuation of the anti-tumor effect of S100A2 exerted by this variant was identified, indicating that asparagine encoded by this variant allele would not compromise the binding capability of S100A2 to target protein(s). Consistent with our *in vitro* studies, no significant alteration of S100A2 protein structure induced by the replacement of serine with asparagine at the residue 62 was observed based on the protein structure modeling (Figure 4). Although the replacement might generate repulsive forces between the amino groups in the asparagine and in phenylalanine at residue 79 or leucine at residue 59 (data not shown), asparagine 62 could rotate freely to minimize the steric effect and subsequently stabilize the protein structure.

This coding sequence variant of S100A2 is originally identified in psoriasis and exists in the two allelic forms, the predominant G allele (94.9%) and the minor A allele (6.1%) in Caucasians (Stoll *et al*, 2001). In 156 chromosomes they examined, there was no significant difference in the allele distribution between normal healthy control and psoriatic individuals. They reported that this nucleotide polymorphism 185G > A had no role in the pathogenesis of psoriasis. Based on dbSNP released by NCBI, this polymorphism (rs1047325) was later reported to have distinct allele frequency for minor S100A2_185A allele in different ethnic groups: 41% in African American, 8% in Europeans; and 2% in Han Chinese (23–24 individuals in each group). Given the allele frequency for A allele is 2% in Han Chinese, we analyzed the S100A2 alleles in 108 chromosomes from both normal and tumor tissues. No A allele could be detected in any of 54 pairwise oral SCC and their adjacent normal tissues. Although S100A2_185G > A is a non-synonymous SNP, there was no significant difference in the allele distribution between normal and oral SCC patients (P = 0. 1552 by Chi-squared test).

Moreover, we could not detect any functional difference between G and A allele in the same locus of S100A2 coding sequence using both molecular and cellular approaches. By contrast, Strazisar *et al* (2009) reported that S100A2_185G > A polymorphism was a tumor-specific alteration in non-small cell lung carcinoma. Although both oral cancer and non-small cell carcinoma are mostly squamous cell carcinoma, S100A2 is frequently overexpressed in lung cancer cells and promotes metastasis (Wang *et al*, 2005; Bulk *et al*, 2009) but the same protein behaves as tumor suppressor in oral cancer cells (Nagy *et al*, 2001; Mueller *et al*, 2005; Tsai *et al*, 2006; Zhang *et al*, 2007). Two contrasting roles of the same protein in different tissue background may explain for differential selection pressure for this coding sequence variation. Together, this coding sequence variation has no regulatory role in S100A2-mediated anti-tumor effect at least in the context of oral cancer.

We also examined if such polymorphism had any effect on S100A2 mRNA or protein expression in cancer cells, we performed RT-PCR and Western blot analyses using five cancer lines with three genotypes at the position 185 along with primary NOKs. No strict correlation in the genotypes of S100A2 gene with the expression level was detected (Figure 1b). Together, codon 62 variation had no impact on the differential expression of S100A2 in oral cancer cells. We also found a non-coding 314T > C polymorphism in the 3′-UTR of S100A2 in CAL-27 and SCC-15 cells with S100A2_185A allele. For unknown reasons, these two sequence variations in the S100A2 gene were co-selected for during evolution. S100A2_314T > C polymorphism has been also found in non-small cell carcinoma (Strazisar *et al*, 2009). More studies are needed to address the function of this non-coding polymorphism and its interplay with coding 185G > A polymorphism.

By comparing published S100A2 protein sequence, man and cow shares 89.7% amino acid identity. The peptide 62 residue is aspartic acid in cow but serine in human. Moreover, the same residue for the putative S100A2 gene in chimpanzee is asparagine, indicating that this codon is not highly conserved during evolution. Although rodents were believed to encode S100A2-like sequence, no definite studies ever showed the existence of S100A2-like protein in these animals. Together, peptide residue 62 in the S100A2 protein is not highly conserved at least in the evolution of primates and cow. Although this amino acid resides in the putative target protein binding site of S100A2, this study indicates that S100A2_185G > A is a neutral coding polymorphism in the aspect of anti-tumor functions mediated by S100A2.

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Conflict of interest statement

The authors state no conflict of interest.

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