Loss of heterozygosity on chromosome 6q correlates with decreased thrombospondin-2 expression in human salivary gland carcinomas

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Since loss of heterozygosity (LOH) on the long arm of chromosome 6q is frequently observed in salivary gland carcinomas, we examined 28 salivary gland carcinomas using 24 microsatellite markers mapping to 6q15-27 to identify the commonly deleted region that we felt might contain one or more tumor suppressor genes. LOH was detected in at least one locus in 10 of 28 tumors (35.7%). The most frequently deleted regions occurred between D6S1581 and D6S305 (LOH cluster region 1 (LCR1) and between D6S297 and D6S1590 (LCR2). LOH was observed in 60% of adenoid cystic carcinomas (ACC) and in 57.1% of mucoepidermoid carcinomas (MEC), but was not observed in any locus in any other histological subtypes studied. The gene encoding for thrombospondin-2 (*TSP-2*) is located in LCR2 and 8 of 9 tumors demonstrating LOH in this region also showed significantly decreased TSP-2 expression by immunohistochemistry. As *TSP-2* is a potent inhibitor of tumor growth and angiogenesis, we examined whether TSP-2 expression correlated to microvascular angiogenesis in these tumors and discovered that microvessel counts were significantly higher in lesions with decreased TSP-2 expression (*P*=0.02). Our results suggest that 6q LOH may be a significant event in salivary gland carcinogenesis, particularly in ACC and MEC, and that the correlated decrease of TSP-2 expression also plays a critical role. (Cancer Sci 2003; 94: 530–535)

Salivary gland carcinomas are relatively rare tumors of the head and neck. These lesions consist of various types of neoplasms with different biological behaviors, including occasional malignant potential. Because of their marked heterogeneity and relative rarity, they continue to pose diagnostic and treatment difficulties.[1,2] The histogenesis of these tumors is an interesting topic and several investigations have demonstrated different patterns of genetic alterations among each histological subtype[3–5]; however, the numerous tumorigenetic mechanisms that have been proposed remain hypothetical, even though it is accepted that molecular genetic alterations are responsible for the initiation and progression of cancers in general.

It is believed that salivary gland carcinomas, like other neoplastic diseases, develop and progress through an accumulation of alterations in proto-oncogenes and tumor suppressor genes (TSGs).[3–6] Identification of TSGs within the chromosomal regions showing frequent allelic losses and deletions is mechanistically critical in determining the multistep process of malignant transformation. Frequent loss of heterozygosity (LOH) in a chromosomal region in a tumor is considered to be an indication of the presence of a putative TSG. LOH on chromosome 6q is a frequent event in a variety of cancers, including those of the prostate,[7,8] breast,[9] ovary,[10] and salivary gland.[4,11,12] Evidence for the presence of a tumor suppressor gene or genes on chromosome 6 comes from a study showing that microcell-mediated chromosome transfer into breast cancer cell lines resulted in suppression of metastatic ability, as well as tumorigenicity.[13] In salivary gland carcinomas, LOH was most frequently observed on chromosomes 1p, 2p, 6q, 12p, and 19q.[3,4] Most LOH studies, however, focused on genome-wide investigations, and detailed deletion mappings were carried out in only a few histological subtypes. LOH analysis using polymorphic microsatellite markers is a method sensitive enough to detect micro-deletions that may otherwise escape detection.

We used microsatellite LOH analyses on samples from 28 salivary gland carcinomas, comprised of 8 histological subtypes, to identify any commonly deleted regions on 6q. We also evaluated the immunohistochemical expression of thrombospondin-2 (TSP-2), a putative TSG that resides within LOH cluster region 2 (LCR2), and looked for evidence of correlation between 6q LOH, TSP-2 expression, and clinicopathological parameters.

Materials and Methods

Tumor samples. Tumor samples were obtained from 28 patients who had undergone radical surgery or biopsy at the Department of Oral and Maxillofacial Surgery at Nara Medical University; the samples consisted of 10 adenoid cystic carcinomas (ACC), 7 mucoepidermoid carcinomas (MEC), 3 squamous cell carcinomas (SCC), 3 acinic cell carcinomas (AC), 2 carcinoma in pleomorphic adenomas (Ca in PA), 1 adenocarcinoma (ADC), 1 salivary duct carcinoma (SDC), and 1 basal cell adenocarcinoma (BCC). None of the patients had undergone preoperative chemotherapy or radiotherapy. Sections from each sample were fixed in 10% neutral buffered formalin, paraffin-embedded, and cut at 5 μm for hematoxylin and eosin (H&E) staining. Equivalent sections were cut at 3 μm and mounted unstained for immunohistochemistry (IHC). Tumor specimens were graded according to the 1991 WHO classification criteria.[14]

DNA extraction. One H&E section of each sample was used as a guide for selecting desired areas of both tumor and normal tissue. The areas selected from the H&E slides were dissected from the corresponding unstained sections using a scalpel and DNA was extracted as previously described.[15] Control DNA was extracted from normal tissues, not adjacent to the cancerous focus to prevent possible sample contamination.

Microsatellite markers and PCR amplification. The primers for specific microsatellite sequences and the chromosomal maps and distances as listed in Fig. 1 were obtained from the Whitehead Institute (http://www-genome.wi.mit.edu/) and the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gor/). A standard PCR reaction was carried out in a 10 μl reaction volume containing 1.5 m*M* $MgCl_2$, 5 μ*M* of each 2 primers (i.e., one 5′-end fluorescently labeled primer and one unlabeled primer), 0.2 m*M* deoxynucleotide triphosphates, 1 μl of DNA, and 0.5 unit of "Platinum" *Taq* DNA

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polymerase (Invitrogen Corp., Carlsbad, CA). After initial denaturation at 94°C for 5 min, 33 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final extension for 7 min.

LOH analysis. Genomic DNA from each sample was analyzed using 24 polymorphic microsatellite markers. In brief, 12 μl of deionized formamide was mixed in a genetic analyzer sample tube with 0.5 μl of GeneScan Internal Lane Size Standard ("ROX", Applied Biosystems, Foster City, CA) and 1μl of PCR product diluted 1:5 or 1:10. The samples were denatured by heating at 94°C for 2 min, then placed in an ice-water bath and size-separated on an automated DNA sequencer ("ABI 310" Genetic Analyzer, Applied Biosystems). LOH can be defined mathematically using the following equation: LOH value=(height of longer normal allele/height of shorter normal allele)/(height of longer tumor allele/height of shorter tumor allele). A tumor is positive for LOH when 35% of a target allele is lost. An LOH value of <0.65 indicates that the tumor sample shows significant loss of the longer allele, whereas an LOH value of >1.5 indicates that the tumor sample shows significant loss of the shorter allele.

Determination of TSP-2 expression. The TSP-2 expression was assessed by IHC. As stated earlier, some paraffin-embedded sections were cut at 3 μm and left unstained. After deparaffinization, the slides were incubated for 1 h at room temperature with a polyclonal anti-TSP-2 antibody (clone N-20; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 for detection of TSP-2 expression, then washed, and reactions were visualized using a Histofine SAB-PO kit (Nichirei, Tokyo) with diaminobenzidine as the chromagen. All sections were counterstained with hematoxylin. Tumors were considered immunonegative for TSP-2 if <5% of cells showed staining.

We determined the expression of mRNA of TSP-2 by RT-PCR and protein expression by western blot analysis. Total RNAs and protein were isolated from case 5 and two normal human salivary glands using RNA TRIzol reagent (Invitrogen Corp.). RT-PCR was carried out in 50 μl containing RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) using the *G3PDH* gene as a reference. The sequences of the primers and the PCR cycle conditions were described previously.[16, 17]

Protein expression was examined by western blot analysis. Immunoblotting was performed as described previously.[18, 19] Briefly, tissue lysates were resolved on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Ltd., Bedford, MA). Membranes were incubated overnight with a polyclonal anti-TSP-2 antibody (clone N-20), washed with Tris-buffered saline-Tween 20, incubated with horseradish peroxidase-conjugated antigoat IgG, and analyzed by the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Vascularization in salivary gland carcinoma. Paraffin-embedded sections were examined immunohistochemically with mouse anti human CD34 monoclonal antibody (Nichirei). For the microvessel evaluation, three regions with the highest microvessel counts at ×200 magnification in each tumor sample were averaged and the averages were compared between samples. Large vessels with thick muscular walls were excluded in these counts.

Statistical analysis. Fisher's exact test was used to analyze LOH and TSP-2 expression against clinicopathological factors. Welch's *t* test was used to analyze TSP-2 expresssion and microvessel counts. A probability value of <0.05 was considered statistically significant.

Results

Chromosome 6q LOH in salivary gland carcinomas. Paired normal tissue and tumor samples from 28 salivary gland carcinomas were screened for LOH on 6q to determine the frequency of allelic imbalance and to define the minimal region of deletion. A total of 24 loci were tested in all of the matched normal/tumor samples; the markers are listed in Fig. 1 and are based on the latest mapping information obtained from the NCBI and Whitehead Institute as previously noted. Fig. 2 shows representative allelic retention, LOH and non-informative in normal tissue versus LOH in tumor tissue in case no. 5. Ten of 28 (35.7%) tumors showed LOH on 6q in at least one locus. As can be seen in Table 1, 6q LOH was detected in 6 of the 10 ACC (60.0%) and 4 of the 7 MEC (57.1%), but was not detected in any other histological type of carcinoma represented.

Frequent LOH was detected in two particular separate loci on 6q (6q25-27). The 2.0 cM DNA segment between microsatellite

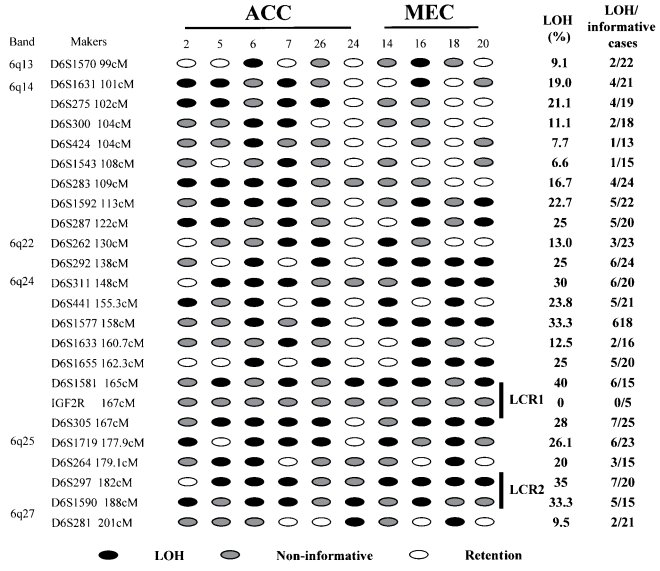

Fig. 1. Schematic representation of LOH mapping on 6q in salivary gland carcinomas. Sample identification numbers and histology are indicated on the top of the figure. The markers analyzed and respective chromosomal locations are shown on the left. Black, gray and unfilled circles indicate LOH, non-informative and retention, respectively. The frequencies of LOH are shown on the right, and bars show LOH cluster regions (LCR).

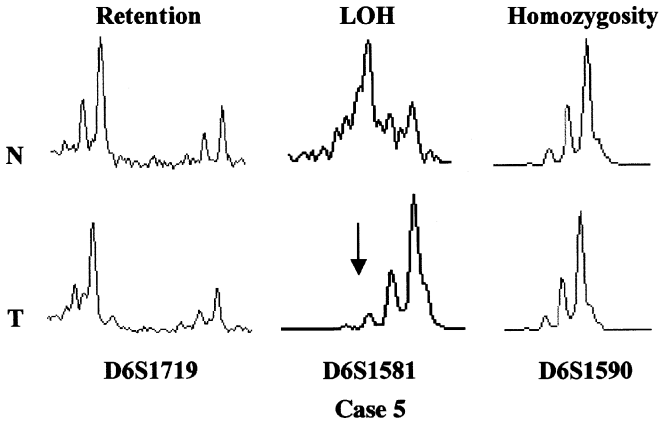

Fig. 2. An example of the analyses of loss of heterozygosity (LOH) in one case (sample no. 5) using the ABI 310 genetic analyzer; comparison of tumor (T) and normal (N) tissues. The arrow points to the lost shorter allele.

Table 1. Pathological diagnosis and frequencies of LOH at 6q25-27 in 28 cases of salivary gland carcinomas

Histopathology diagnosis	No. of cases	LOH (+)	(%)
Adenoid cystic carcinoma (ACC)	10	6	60.0
Mucoepidermoid carcinoma (MEC)	7	4	57.1
Acinic cell carcinoma (AC)	3	0	0
Squamous cell carcinoma (SCC)	3	0	0
Carcinoma in pleomorphic adenoma (Ca in PA)	2	0	0
Basal cell adenocarcinoma (BCC)	1	0	0
Salivary duct carcinoma (SDC)	1	0	0
Adenocarcinoma (ADC)	1	0	0

Table 2. LOH on 6q25-27, TSP-2 expression and clinicopathological parameters

Case	Age	Gender	Diagnosis	Site	Stage	LOH 6q25-27	TSP-2 expression
1	67	F	ACC	MG	III, IV	–	+
2	50	M	ACC	MG	III, IV	LOH	–
3	80	F	ACC	SL	III, IV	–	–
4	44	F	ACC	MG	III, IV	–	–
5	53	F	ACC	MG	III, IV	LOH	–
6	77	M	ACC	MG	III, IV	LOH	–
7	48	F	ACC	SM	I, II	LOH	+
8	71	F	ACC	P	III, IV	–	–
9	72	F	AC	P	I, II	–	–
10	54	F	ADC	P	I, II	–	+
11	73	M	BCC	SM	I, II	–	–
12	47	M	Ca in PA	P	III, IV	–	+
13	49	M	Ca in PA	MG	I, II	–	+
14	16	F	MEC	MG	I, II	LOH	–
15	65	F	MEC	P	I, II	–	+
16	60	M	MEC	SM	III, IV	LOH	–
17	15	M	SCC	P	I, II	–	+
18	74	F	MEC	SM	III, IV	LOH	–
19	53	M	SCC	SM	III, IV	–	+
20	74	F	MEC	SM	III, IV	LOH	–
21	58	M	SDC	P	III, IV	–	+
22	67	M	MEC	P	III, IV	–	–
23	55	M	AC	SM	I, II	–	–
24	44	F	ACC	SM	III, IV	LOH	–
25	34	M	AC	P	I, II	–	–
26	66	M	ACC	MG	I, II	LOH	–
27	84	F	MEC	P	III, IV	–	–
28	75	M	SCC	P	III, IV	–	+

ACC, adenoid cystic carcinoma; MEC, mucoepidermoid carcinoma; AC, acinic cell carcinoma; Ca in PA, carcinoma in pleomorphic adenoma; SDC, salivary duct carcinoma; ADC, adenocarcinoma (NOS); SCC, squamous cell carcinoma; BCC, basal cell adenocarcinoma; P, parotid; SM, submandibular; SL, sublingual; MG, minor gland.
Tumors were considered negative for TSP-2 expression when <5% of cancer cells showed staining.

markers D6S1581 and D6S305 was lost in 9 of 25 (36.0%) informative specimens and we designated this segment as LCR1. The second locus, a 6.6 cM segment flanked by markers D6S297 and D6S1590, was lost in 9 of 23 (39.1%) informative specimens and was designated LCR2 (Fig. 1). LCR1 and LCR2 are separated by a distance of 15 cM on chromosome 6q according to data provided by the Whitehead Institute. The *M6P/IGF2R* gene is located in the vicinity of LCR1 (0.2 cM centromeric to D6S305); we further discovered that all specimens with LOH on LCR1 were homozygous for the *M6P/IGF2R* marker (Fig. 1).

Correlation to clinicopathological parameters. We compared LOH at 6q25-27 and with clinicopathological parameters including age, gender, tumor site, survival probability and stage in all tumors examined. We were unable, however, to find any statistically significant correlation between these variables (Table 2).

Table 3. LOH on LCR2 and TSP-2 IHC expression

TSP-2 expression	LOH (+)	LOH (–)	*P*
Positive (+)	1	8	0.03
Negative (–)	8	6	

TSP-2 expression. Immunohistochemical detection of TSP-2 expression was observed in only 10 of 28 salivary gland carcinomas (35.7%), whereas TSP-2 was expressed in all samples of normal salivary gland. In addition, 8 cases demonstrating LOH in LCR2 were immunonegative for TSP-2 expression; only 1 tumor with LOH in LCR2 was also positive for TSP-2 expression (P=0.03, Table 3). Reduced expression of TSP-2 in ACC (case 5) relative to normal parotid and submandibular glands was validated by RT-PCR (Fig. 3A). A 180-kDa protein was

detected as a single specific band by western blot analysis (Fig. 3B). This sample also exhibited decreased TSP-2 expression on immunohistochemistry (Fig. 4). When compared to other histological subtypes, ACC and MEC showed significantly decreased TSP-2 expression (P=0.03, Table 4). We found no statistically significant correlation between TSP-2 expression and clinicopathological factors (Table 2).

Association between TSP-2 expression and microvessel counts. The mean microvessel count among tumor samples was 47.9 at ×200 magnification (range 12–138). The mean microvessel count in TSP-2-positive lesions was 33.8±15.6, while that in TSP-2-negative cancers was 55.8±32.4, demonstrating a significant inverse correlation of TSP-2 expression with decreased microvessel counts in salivary gland carcinomas (P=0.02, Table 5).

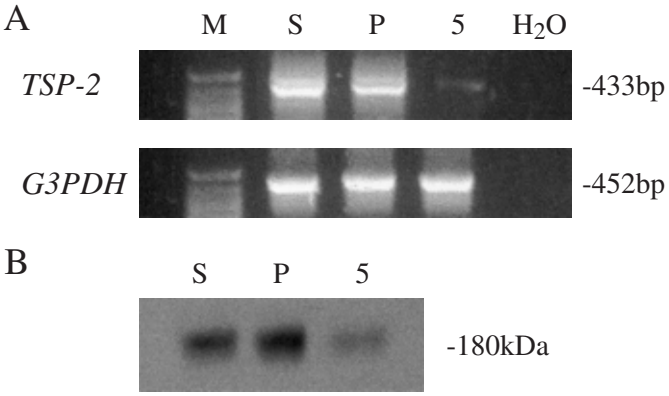

Fig. 3. The expression of TSP-2 in normal salivary glands and case 5 (S, submandibular gland; P, parotid gland; 5, case 5). (A) In both normal salivary glands, mRNA of TSP-2 was clearly detected by RT-PCR analysis and markedly reduced expression was observed in case 5. (B) A single band of 180-kDa protein was detected by western blotting.

Discussion

Previous comparative genomic hybridization (CGH) and LOH studies of salivary gland carcinomas have indicated deletion of 6q in up to 75% of samples examined.[11, 12, 20] We have been able to more precisely define the location of allelic losses within the 6q25-27 chromosomal regions, demonstrating two "hotspots" or LOH cluster regions in a subset of tumors we evaluated. One region, designated LCR1, is comprised of a 2.0 cM DNA segment flanked by microsatellite loci D6S1581 and D6S305; the second region, LCR2, occurs in a 6.6-cM region between marker loci D6S297 and D6S1590. Queimado *et al*. also reported two commonly deleted regions in salivary gland carcinomas, one between D6S262 and D6S32, and another between D6S297 and D6S446.[11] While we did not find the former region to be frequently affected, the latter segment over-

Table 4. TSP-2 IHC expression and tumor subtype

Tumor subtype	TSP-2 (+)	TSP-2 (–)	*P*
ACC and MEC	3	14	0.03
All others	7	4	

Table 5. TSP-2 IHC expression and microvessel counts

TSP-2 expression	*n*	Vessel counts Mean±SD	*P*
Positive (+)	10	33.80±15.64	0.02
Negative (–)	18	55.78±32.39	

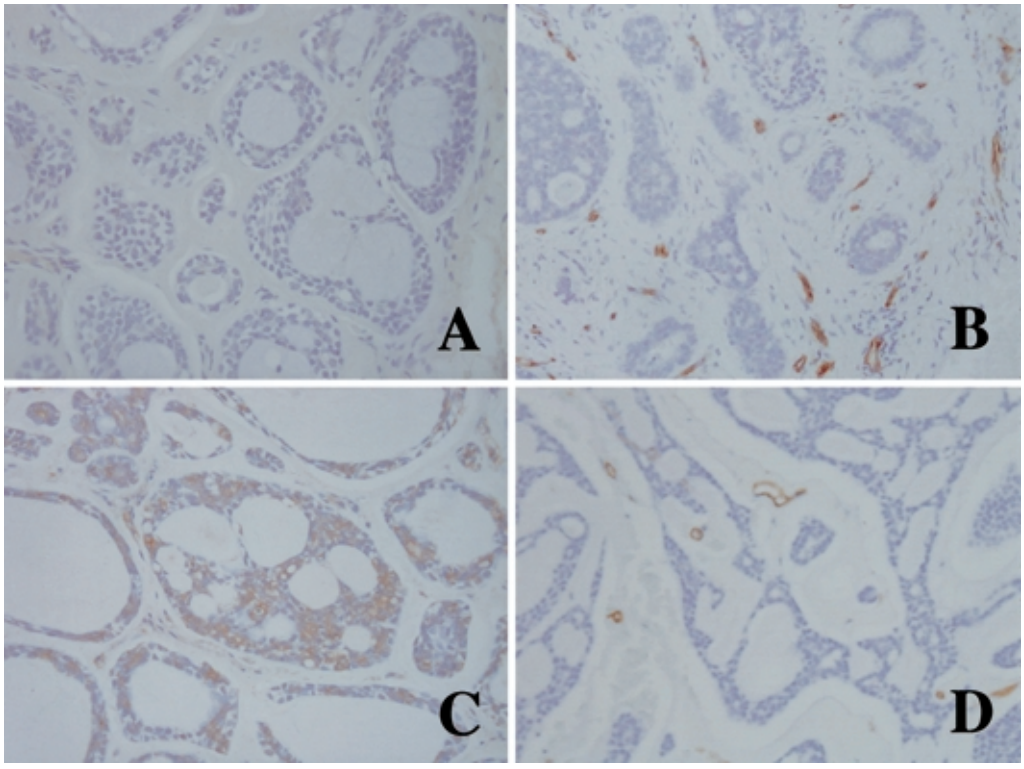

Fig. 4. Immunohistochemical staining for TSP-2 and CD34. (A) Lack of TSP-2 immunoreactivity in adenoid cystic carcinoma (ACC; sample no. 5). This case showed significantly increased vascular density. (B) A second ACC (sample no. 1) exhibits cytoplasmic immunoreactivity for TSP-2 in a majority of cancer cells (C) as well as decreased vascular density (D).

laps our LCR2. Some investigators have presented evidence that there are different patterns of LOH among each histological type;[3, 4, 21] several studies found frequent 6q deletions specifically in ACC, whereas a few studies reported common karyotypic alteration on 6q in other types of salivary gland carcinomas. We can corroborate some of these findings, in that we similarly detected 6q LOH in 6 of 10 (60%) ACC and in 4 of 7 (57.1%) MEC, while LOH was not detected in other types of salivary carcinomas. This study lends weight to the suggestion that different patterns of LOH, possibly even within conserved LCRs, are responsible for the histogenetic variability of salivary gland tumors.

These LCRs are likely to harbor candidate TSGs, inhibition of which may be necessary for development of salivary gland carcinoma. Cerilli *et al*. screened five types of salivary gland carcinomas for 9p21 deletions and *p16* mutations; they found frequent genetic alterations in only one subtype, and different genetic mechanisms of TSG inactivation in each histological type, of salivary gland carcinoma.[5] This study also showed significantly decreased TSP-2 expression specifically in salivary ACC and MEC as compared with other histological subtypes. Abnormalities in the two regions singled out in this study, LCR1 and LCR2, have been detected in several malignancies and several genes that potentially functional in tumorigenesis have been localized in the vicinity of both. The gene encoding mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6P/IGF2R*) is located in the vicinity of LCR1; this receptor is multifunctional, being required for the activation of the growth inhibitor, transforming growth factor β1 (TGFβ1), for the degradation of the mitogen IGF2, and for the intracellular sorting of lysosomal enzymes.[22, 23] *M6P/IGF2R* has further been shown to suppress cancer cell growth,[24] and to also be mutated in a number of human cancers, including those that develop in the liver,[25] breast,[26] and lung.[27] *M6P/IGF2R* appears to be an imprinted gene in mice,[28] rats,[29] and in a subset of human populations.[30] In this study, 9 of 25 informative tumors showed LOH at this region. As in other types of human cancers, inactivation of the second allele, possibly by methylation, may initiate tumorigenesis in the salivary gland.

The gene encoding *TSP-2* is located in the LCR2. TSP-2 is a matricellular glycoprotein and a potent inhibitor of tumor growth and angiogenesis.[31, 32] Tumors are believed to secrete a variety of angiogenic factors, the presence of which has been shown to be useful for prediction of metastasis in several cancers[33]; indeed, quantification of angiogenesis, as measured by microvessel counts, can be used as a prognostic factor in malignant tumors.[34, 35] As a process, angiogenesis is regulated by the balance of a variety of angiogenic stimulators and inhibitors. TSP-2 expression has been reported to be significantly correlated with inhibition of angiogenesis and metastasis in some cancers,[31, 36] while other studies were unable to show any correlation of TSP-2 with angiogenesis and/or prognosis.[37] This does not preclude the possibility that the relevance of TSP-2 expression and tumor angiogenesis may be dependent on individual tumor histology and organ peculiarity.

We demonstrated that LOH in LCR2 is significantly correlated with decreased TSP-2 expression. RT-PCR and western blot analysis demonstrated the reduced expression of TSP-2 in case 5, which showed LOH on LCR2. This sample was also immunonegative for TSP-2 by immunohistochemistry. In addition, the decreased TSP-2 expression is closely correlated with increased tumor vascularity in salivary gland carcinoma. Streit *et al*. demonstrated that when human squamous cell carcinoma cell lines, which do not normally express TSP-2, were transfected with a TSP-2 expression vector, the transfected cells did not show an altered growth rate or colony-forming ability and were susceptible to induction of apoptosis.[38] This would imply that TSP-2 can function as an antiangiogenic moiety and might be a candidate for gene therapy in salivary gland carcinoma, especially in ACC and MEC. An angiogenic phenotype, which may be able to support tumorigenicity, can arise in a stepwise fashion in response to both a decrease in the secretion of inhibitors and the sequential up-regulation of the secretion of angiogenic inducers. We suggest that, at least in a large number of cancers of the salivary gland, the close correlation between TSP-2 expression and microvessel density is critical for tumor progression.

In this study, we failed to detect a significant correlation between tumor angiogenesis and LOH on LCR2. Decreased expression of TSP-2 was observed in 6 of 14 tumors without LCR2 alterations, suggesting that the altered expression of TSP-2 in salivary gland carcinoma might result not only from allelic loss, but also from other genetic and/or epigenetic alterations such as point mutations, promoter methylation or aberrant mRNA splicing. Further work is needed to elucidate the inactivation mechanisms of this gene with larger numbers of these tumors.

In summary, we examined the presence of two relatively specific LCRs on 6q25-27 in salivary gland carcinomas, and showed that LOH occurs more frequently in these LCRs in the ACC and MEC subtypes. Moreover, LOH in LCR2 is closely correlated with decreased TSP-2 expression with an additional significant inverse correlation between TSP-2 expression and decreased vascularity. We think it likely that LOH in LCR2 and decreased TSP-2 expression play a critical role in carcinogenesis of the salivary gland, and that our results provide additional data supporting the idea that morphologically different carcinomas have dissimilar genetic mechanisms of initiation or progression, which may ultimately contribute to differences in their clinical behavior.

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