Expression of angiogenin, TGF-β, VEGF, APEX and TNF-α in oral squamous cell carcinoma

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Abstract

Purpose: The purpose of this study was to verify that the expressions of angiogenin, transforming growth factor-beta(TGF-β), vascular endothelial growth factor(VEGF), human apurinic/apyrimidinic endonuclease(APEX) and tumor necrosis factor-alpha(TNF-α) were associated with the tumorigenesis of the oral squamous cell carcinoma(OSCC).

Materials and Methods: Fifty-one samples of OSCC and fifteen normal oral mucosae were obtained to analyze the expression levels of above five factors. mRNA expressions were quantified by the quantitative competitive PCR(QC-PCR) method. After 2% agarose gel electrophoresis stained with ethidium bromide, the concentration of mRNA was calculated by a digital image analysis system. The expression levels of angiogenin, TGF-β, VEGF, APEX and TNF-α were compared by unpaired Student's t-tests between cancer and normal tissues. We analyzed statistically to find the cut-off values that would be useful as diagnostic markers, and the linear regression analysis between every two factors of these five factors by SAS system.

Results: All of these five factors (angiogenin: P<0.0037, TGF-β: P<0.0001, VEGF: P<0.0102, APEX: P<0.0023, TNF-α: P<0.0074) were significantly correlated with OSCC. In the analysis to find the cut-off values for the diagnosis, we could not find any value that had a reasonable sensitivity and specificity. In the linear regression analysis, there were correlations between angiogenin and TNF-α, TGF-β and VEGF, TGF-β and APEX, TGF-β and TNF-α, VEGF and APEX, VEGF and TNF-α, APEX and TNF-α.

Conclusion: Our results suggest that not only angiogenin, TGF-β, VEGF, APEX and TNF-α are significantly associated with the tumorigenesis, but also the close relationship between these factors might enhance the tumorigenesis of OSCC. We can not find clinical availability for diagnosis.

Key words

Angiogenin, Transforming growth factor-beta(TGF-β), Vascular endothelial growth factor(VEGF), Human apurinic/apyrimidinic endonuclease(APEX), Tumor necrosis factor-alpha(TNF-α), Oral squamous cell carcinoma(OSCC)

INTRODUCTION

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen, and removing waste products. The process of angiogenesis plays an important role in many physiological and pathological conditions.

The first human tumor derived protein with *in vivo* angiogenic activity to be obtained in pure form has been isolated from serum-free supernatants of an established human adenocarcinoma cell line(HT-29) and named angiogenin. It was later found in normal human serum and its mRNA was found to be expressed in human tumor cells[3,4] as well as non-malignant cells such as peripheral blood cells, T cells, fibroblasts, and colon epithelial cells[4]. Because of its capability of tumor angiogenesis, it has since been identified as a potentially important target for anticancer therapy. Angiogenin mRNA and/or protein was elevated in many types of cancer lesions compared with the corresponding non-neoplastic tissues[6,7]. In some cases, high tissue angiogenin levels were correlated with cancer progression or poor prognosis[7-9].

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VEGF, which is also called vascular permeability factor (VPF), is an endothelial cell mitogen that is angiogenic *in vivo*[12,13]. Its expression correlated with blood vessel growth during embryogenesis, and angiogenesis in the female reproductive tract[14] and tumors. Also VEGF induces fenestrations in endothelium of small venules and capillaries and even in tissues where microvessels are not normally fenestrated[15]. Expression of VEGF increases angiogenesis and tumor growth, and anti-VEGF antibodies inhibit tumor growth and reduce experimental metastasis in nude mice. Patients with various histological types of cancer have been found to have elevated serum VEGF concentrations compared to healthy controls[16,17].

Faithful maintenance of the genome is crucial to the individual and to species. DNA damage arises from both endogenous sources such as water and oxygen, and exogenous sources such as sunlight and tobacco smoke. In human cells, base alterations are generally removed by excision repair pathways that counteract the mutagenic effects of DNA lesions[18,19]. APEX is a multifunctional DNA repair enzyme and plays a key role in the DNA base excision repair process. APEX can cleave the DNA phosphodiester backbone immediately 5' to an AP-site. In addition to its endonuclease activity, it also possesses 3' -phosphatase, 3' -phosphodiesterase, RNase H, and 3' -5' -exonuclease activities. APEX knockout mice die in the early embryonic stage[20], which indicates this protein is critical for development. It is also discovered that APEX has a significant preference for the removal of mispaired nucleotides from the 3' terminus of DNA when compared with matched pairs. Because physical interaction between polymerase-β and APEX had been established and APEX showed a significant preference for 3' mispaired nicked DNA[21]. APEX could be the proofreading enzyme correcting the misincorporations introduced by DNA polymerase-β. It is known that substantial elevation of Ap endo activity (13-fold, on average), as well as APEX levels, is characteristic of adult glial tumors. A clinically important consequence of the increased Ap endo activity in malignant gliomas may be enhanced resistance to alkylating agent-based chemotherapy. Oxidative stress induces elevation of APEX protein and Ap endo activity, and is accompanied by increased resistance to these alkylators. Importantly, reduced resistance is accompanied by increased levels of abasic sites, and enhanced resistance is accompanied by decreased abundance of abasic sites, indicating that the apurinic/apyrimidinic site incision activity of APEX is at least partially responsible for drug resistance[22]. Usually the expressions of APEX are closely related with tumor progression and have a diagnostic value. APEX expression and function may not be equivalent in all cell types and tissues, but it is generally accepted that the expression of APEX is correlated with tumor.

TNF-α was first found in studying "hemorrhagic necrosis" of tumors produced by endotoxin[23]. It was found that the serum of Bacillus Calmette-Guerin (BCG)-infected mice treated with endotoxin contained a substance (tumor necrosis factor; TNF) which mimiced the tumor necrotic action of endotoxin itself. TNF-positive serum is as effective as endotoxin itself in causing necrosis of the sarcoma and other transplanted tumors. A variety of tests indicate that TNF-α is not residual endotoxin, but a factor released from host cells, probably macrophages, by endotoxin. Genotypic changes of TNF-α promoter region are associated with endometrial cancer[24]. TNF-α gene encodes a cytokine involved in angiogenesis and oncogenesis of cancers. The genotype changes at -308 promoter region of TNF-α may play an essential role in the malignant transformation.

TGF-β is a potent growth inhibitor of all epithelia and can also induce apoptosis[25,26]. Although TGF-β can be tumor suppressive, there is increasing evidence that TGF-β secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression. TGF-β acts early as a tumor suppressor, probably by inhibiting the proliferation of nontransformed cells, and it acts later as a tumor promoter by eliciting an epithelial-to-mesenchymal transition[27]. The progression of epithelial tumors to an invasive metastatic state is often associated with epithelial-to-mesenchymal transition, downregulation of cellular adhesion molecules, elevated expression of metalloproteases, and increased motility and angiogenesis, all of which can be modulated by TGF-β. It is therefore not surprising that the TGF-β can also promote tumorigenesis by modulating these critical processes.

Many factors are involved in the development and growth of tumor. Angiogenin, TGF-β, VEGF, APEX and TNF-α are well known factors that may be correlated with tumorigenesis. Many studies have shown the correlations between the expressions of these five factors and cancers. Among them, some results demonstrated that the expression levels were correlated with tumor progression. OSCC is one of the most common head and

neck cancers and generally has a poor prognosis due to its tendency towards local invasion and subsequent metastasis, which is mediated by multiple proteolytic enzyme and angiogenesis. If there were simple and reliable diagnostic markers, these would be very helpful to detect OSCC in early stage. There are many studies to concern these factors respectively but it is very rare to study these factors simultaneously. We wanted to confirm the results that showed the correlations between the expressions of these five factors and tumor.

MATERAIALS AND METHODS

Fifty-one OSCC tissues were obtained during operation or at biopsy in the Department of Oral and Maxillofacial Surgery and Otolaryngology, Chungbuk National University Hospital, Cheongju, Chungbuk, Korea. Fifteen normal oral mucosae were obtained from non-cancer patients. Each sample was examined histopathologically and only these that consisted of normal squamous epithelial cells were analyzed. Samples were snap-frozen in liquid nitrogen immediately after excision and stored at -70℃ until RNA extraction. Total RNA was extracted from the cancer tissues and normal oral mucosae by the method of Chomczynski and Sacchi. Complementary DNA (cDNA) was then prepared from 1 μg of total RNA by random priming method using the First-Strand cDNA Synthesis kit (Amersham Biosciences, Buckinghamshire, UK). A target fragment of native cDNA was first obtained by PCR amplification of cDNA from normal oral mucosa with a regular 5' primer. The product was separated by 2% agarose gel electrophoresis. The cDNA was extracted from the gel, then purified with an agarose gel extraction kit (Bioneer, Chungbuk, Korea). To construct a competitive cDNA fragment, the floating primer sequence contained the deletion or insertion of neucleotides. An add-on sequence in the floating primer was designed to contain a specific restriction site. After each set of PCR with the regular 5' primer and the 3' floating primer, and with the regular 3' primer and the 5' floating primer, a competitive cDNA fragment was obtained by PCR of a mixture of the two PCR products with the regular primer set. The PCR mixture contained 2 μl cDNA, 0.5 μl competitive cDNA(0.3 fmol), 10 pmol of each regular primer, 1.25 mM dNTP, 10 X buffer and 0.25 units of Taq DNA polymerase in a total volume of 25 μl(Table 1).

After 2% agarose gel electrophoresis and staining with ethidium bromide, the gel blot was analyzed by a digital image analysis system (DigiFrog, Corebio System, Daejun, Korea)(Fig. 1-5). The logarithmically transformed ratios of target cDNA to competitive cDNA were plotted against the log amount of initially added competitive cDNA in each PCR to obtain a linear reproducible

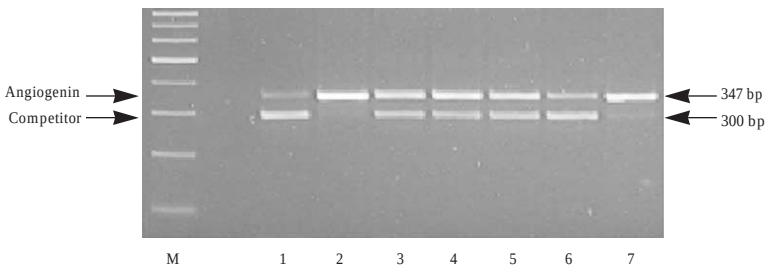

Fig. 1. Examples of the QC-PCR products of the tissue samples and competitor for angiogenin mRNA after electrophoresis in 2% agarose gel and staining with ethidium bromide. Competition occurs between the sample and competitor genes in the PCR reaction. Lane 3 is an example of the QC-PCR product for nearly equal concentration of the sample and competitor. Lanes 2, 4 and 7 are examples of the products for a higher ratio of sample/competitor, and Lanes 1, 5 and 6 are examples of the lower ratio. M: size marker (100 bp ladder).

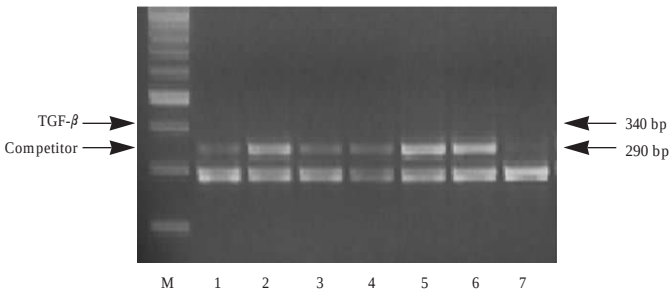

Fig. 2. Examples of the QC-PCR products in TGF-β mRNA after electrophoresis in 2% agarose gel and staining with ethidium bromide. Competition occurs between the sample and competitor genes in the PCR reaction. Lane 5 is an example of the QC-PCR product for nearly equal concentration of the sample and competitor. Lanes 1, 2, 3, 4, 6 and 7 are examples of the products for a lower ratio of sample/competitor. M: size marker (100 bp ladder).

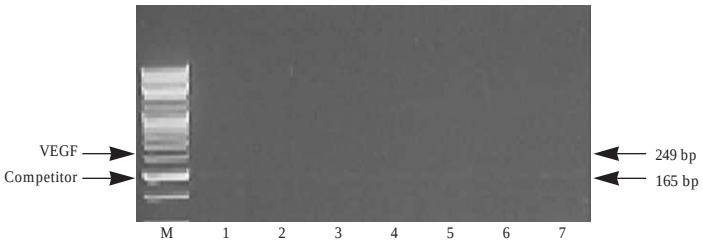

Fig. 3. The QC-PCR findings of VEGF. Competition occurs between the sample and competitor genes in the PCR reaction. Lane 6 is example of the QC-PCR product for nearly equal concentration of the sample and competitor. Lanes 1, 3, 4, 5 and 7 are examples of the products for a higher ratio of sample/competitor, and lane 2 is an example of the lower ratio. M: size marker (100 bp ladder).

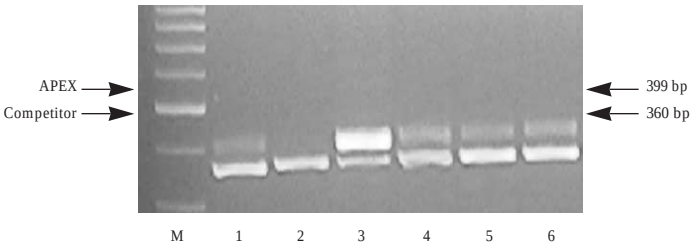

Fig. 4. Examples of the QC-PCR products of the tissue samples and competitor for APEX. Competition occurs between the sample and competitor genes in the PCR reaction. M: size marker (100 bp ladder).

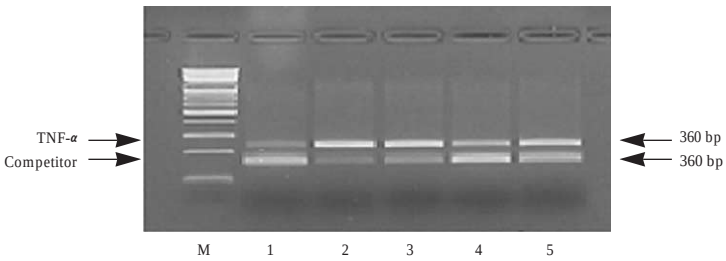

Fig. 5. The QC-PCR findings of the tissue samples and competitor for TNF-α. Competition occurs between the sample and competitor genes in the PCR reaction. Lane 5 is example of the QC-PCR product for nearly equal concentration of the sample and competitor. Lanes 2 and 3 are example of the products for a higher ratio of sample/competitor, and lanes 1 and 4 are examples of the lower ratio. M: size marker (100 bp ladder).

standard curve. Aliquots of 0.3 fmol of competitive cDNA were added to each unknown sample before PCR. The ratios of the densities of the target cDNA to competitive cDNA were ca1culated. Quantitative competitive PCR(QC-PCR) was carried out on at least two aliquots of the cDNA of each sample as described earlier, and the results did not differ by more than 5%. The expression levels of angiogenin, TGF-β, VEGF, APEX, TNF-α were compared by unpaired Student's t-test between cancers and normal tissues. We took the analysis to find the cut-off values that would be used as reasonable diagnostic markers between cancer and normal tissues. We performed that analysis by midpoint between the mean of cancer and normal tissues, and by 70% of midpoint and 60% and 50%. We took the linear regression analysis between every two factors of these five factors. The SAS package(version 8.01) was used, and $P<0.05$ was considered to be statistically significant.

Table 1. Nucleotide primers and PCR product sizes and PCR conditions

	angiogenin
5' primer	ACT CCA GGT ACA CAC ACT TC
3' primer	TGA CTG ATC CAA GTG GAC CG
The size of PCR product	347bp
5' primer and the 3' floating primer (QC 3' primer)	CTC TGC GAG GGT TTC CGG ATC CGT GTT GAT GTC TTT GCA GGG
3' primer and the 5' floating primer (QC 5' primer)	GCA AAG ACA TCA ACA CGG ATC CGG AAA CCC TCA CAG AGA AAA
The size of PCR product	300bp
The PCR conditions	1 cycle at 94℃ for 5 minues followed by 35 cycles of 30 seconds at 94℃, 30 seconds at 62℃, and 30 seconds at 72℃. The reaction was terminated at 72℃ for 10 minutes and was quenched at 4℃.
	TGF-β
5' primer	AGC TAT CCA CCT GCA AGA CT
3' primer	TCT CGG AGC TCT GAT GTG TT
The size of PCR product	340bp
5' primer and the 3' floating primer (QC 3' primer)	GAC CTC CTT GGC GTA GTA GGA TCC TGC TGT TGT ACA GGG CGA
3' primer and the 5' floating primer (QC 5' primer)	TCG CCC TGT ACA ACA GCA GGA TCC TAC TAC GCC AAG GAG GTC
The size of PCR product	290bp
The PCR conditions	1 cycle at 94℃ for 5 minutes followed by 40 cycles of 30 seconds at 94℃, 30 seconds at 57℃, and 30 seconds at 72℃. The reaction was terminated at 72℃ for 10 minutes and was quenched at 4℃.
	VEGF
5' primer	GTG AAG TTC ATG GAT GTC
3' primer	ATA TCT CCT ATG TGC TGG
The size of PCR product	249bp
5' primer and the 3' floating primer (QC 3' primer)	TGT TGG ACT CCT CAG TGG ATC CCG ATC TCA TCA GGG TA
3' primer and the 5' floating primer (QC 5' primer)	TCA CCT GAT GAG ATC GGG ATC CAC TGA GGA GTC CAA CA
The size of PCR product	165bp
The PCR conditions	1 cycle at 94℃ for 5 minutes followed by 35 cycles of 30 seconds at 94℃, 30 seconds at 58℃, and 30 seconds at 72℃. The reaction was terminated at 72℃ for 10 minutes and was quenched at 4℃.
	APEX
5' primer	GAT CTG CTC TTG GAA TGT GG
3' primer	AGG AAC TTG CGA AAG GCT TC
The size of PCR product	399bp
5' primer and the 3' floating primer (QC 3' primer)	CTG TAC CCT TCC TTG TGG ATC CGA AGT TCA GCT GGT AG
3' primer and the 5' floating primer (QC 5' primer)	CTA CCA GCT GAA CTT CGG ATC CAC AAG GAA GGG TAC AG
The size of PCR product	360bp
The PCR conditions	1 cycle at 94℃ for 5 minutes followed by 40 cycles of 30 seconds at 94℃, 30 seconds at 59℃, and 30 seconds at 72℃. The reaction was terminated at 72℃ for 10 minutes and was quenched at 4℃.
	TNF-α
5' primer	AAA GCA TGA TCC GGG ACG TG
3' primer	AAG ATG ATC TGA CTG CCT GG
The size of PCR product	231bp
5' primer and the 3' floating primer (QC 3' primer)	CAG GCA GAA GAG CGG GAT CCG GGC CCC CCT GTC TT
3' primer and the 5' floating primer (QC 5' primer)	AGA CAG GGG GGC CCG GAT CCC GCT CTT CTG CCT GC
The size of PCR product	173bp
The PCR conditions	1 cycle at 94℃ for 5 minutes followed by 35 cycles of 30 seconds at 94℃, 30 seconds at 50℃, and 30 seconds at 72℃. The reaction was terminated at 72℃ for 10 minutes and was quenched at 4℃.

RESULTS

The mRNA expressions of 51 tumor and 15 normal mucosal samples were assessed by QC-PCR and the gel blots were analyzed by a digital image analysis system. The mean age of cancer patients was 58.7 ± 9.9 with range 35-82 and normal controls was 24.8 ± 10.8 with range 17-40. There was a large difference between the mean age of cancer patients and normal controls. To eliminate the possibility that the expression difference between them would be from the effect of age, we performed t-tests between the group which member's age was older than the mean and the group younger than the mean. T-tests were performed for angiogenin, TGF-β, VEGF, APEX and TNF-α of cancer patients and normal controls, respectively. There were no significant differences by the age of samples (p values ranged from 0.21 to 0.99).

1. mRNA expression of angiogenin, TGF-β, VEGF, APEX and TNF-α

1) mRNA expression of angiogenin

In OSCCs and normal oral mucosae, the mean ± standard deviation values of mRNA expression of angiogenin were 98.67 ± 121.93 pg/ml and 39.73 ± 32.76 pg/ml respectively. The expression value of angiogenin of OSCCs was about 2.5 times higher than normal controls(P<0.0037).

2) mRNA expression of TGF-β

The mean ± standard deviation values of OSCCs and normal oral mucosae were 48.48 ± 61.64 pg/ml and 8.62 ± 6.65 pg/ml, respectively. The expression value of TGF-β of OSCCs was about 6 times higher than normal controls(P<0.0001).

3) mRNA expression of VEGF

OSCCs had about 3 times higher VEGF levels than normal controls. The mean ± standard deviation values of OSCCs and normal oral mucosae were 9.20 ± 13.21 pg/ml and 3.31 ± 4.78 pg/ml, respectively(P<0.0102).

4) mRNA expression of APEX

OSCCs had about 2 times higher APEX levels than normal controls. The mean ± standard deviation values of OSCCs and normal oral mucosae were 30.96 ± 40.05 pg/ml and 14.08 ± 9.84 pg/ml, respectively(P<0.0023).

5) mRNA expression of TNF-α

The mean ± standard deviation values of OSCCs and normal oral mucosae were 4.34 ± 4.04 pg/ml and 2.55 ± 1.23 pg/ml, respectively. The difference of the expression of TNF-α between OSCCs and normal contols was still significant, but the difference of TNF-α between them was the least significant value among five factors in our study(P<0.0074).

We performed t-tests of five factors and got results that all of five factors showed significant differences between cancer tissues and normal controls(Table 2, 3, 4).

2. Cut-off value

We analyzed the mRNA expression levels of angiogenin to find the useful cut-off values for the diagnosis by midpoint between the mean of cancer and normal tissues, and by 70% of midpoint and 60% and 50%. The midpoint between OSCCs and normal controls was 68.95. The sensitivity and specificity were 41% and 82%, so they were considered not useful. The 70% of midpoint was 59.1. The sensitivity and specificity were 46% and 82%. They were considered not useful, either. We were looking for cut-off values at the 60% of midpoint and 50%, but there were no useful values at any point(Table 4).

3. Linear regression analysis

We analyzed statistically the correlations between every two factors of these five factors. There were correlations between angiogenin and TNF-α, TGF-β and VEGF, TGF-β and APEX, TGF-β and TNF-α, VEGF and APEX, VEGF and TNF-α, APEX and TNF-α. Angiogenin and TGF-β, angiogenin and VEGF, angiogenin and APEX showed no correlations between them(Table 5).

DISCUSSION

In the early 20th century, Lewis observed that the vascular architecture depended on the tumor type, and proposed that the cancer environment determined the growth and morphological characteristics of tumor vessels. Tumor vascularization is a vital process for the progression of all solid tumors from a small, localized focus to a large tumor with the capability to metastasis[32]. This progression begins with the sprouting or intussusception from pre-existing host vessels. Circulating endothelial

Table 2. The expression levels of angiogenin, TGF-β, VEGF, APEX, TNF-α of cancer patients and normal controls

No.	SEX	AGE	angiogenin (40 pg/ml)	TGF-β (50 pg/ml)	VEGF (4 pg/ml)	APEX (45 pg/ml)	TNF-α (5 pg/ml)
1	M	62	124	9.97	1.11	2.83	1.54
1	M	61	28	48.49	4.94	24.00	1.67
1	M	48	508	18.23	2.56	67.46	4.79
1	M	55	584	35.47	15.12	92.10	11.80
1	F	70	96	66.24	6.90	41.23	6.95
1	M	72	76	47.12	12.82	45.37	2.98
1	M	52	60	123.73	6.04	39.47	6.53
1	M	64	364	47.40	45.32	56.92	3.62
1	M	67	84	9.19	2.63	6.55	1.65
1	M	56	12	12.13	0.53	1.20	0.94
1	F	66	156	134.67	22.68	52.40	3.60
1	M	67	20	134.74	8.87	68.99	3.15
1	M	54	48	9.46	3.20	6.82	1.70
1	M	58	132	67.86	3.40	17.46	1.31
1	M	59		1.57	0.82	6.19	1.83
1	M	73	132	14.39	0.83	5.58	1.77
1	F	76	120	5.45	0.00	0.29	1.95
1	M	46	128	61.48	7.27	58.71	1.07
1	M	60	20	20.96	3.66	4.53	0.92
1	F	56	40	35.12	6.04	29.91	2.70
1	M	60	36	6.28	3.07	0.64	1.98
1			196	17.93	6.20	9.58	2.56
1	M	78	28	81.31	3.09	19.10	1.73
1	M	61	48	90.01	9.30		2.25
1	M	63	16	5.50	1.94	1.22	2.91
1	M	64	20	24.94	3.16	2.11	2.45
1	F	53	8	3.46	0.58	1.13	2.14
1			76	9.14	0.62	0.39	3.28
1	F	82	8	18.57	2.30	4.65	2.26
1	F	42	12	1.93	0.31	1.43	2.27
1	F	58	64	19.29	10.95	33.48	3.71
1	M	59	16	16.73	1.00	2.73	1.82
1	M	56	36	32.16	6.85	9.94	2.20
1	M	65	68	22.63	7.73	13.87	2.05
1	F	58	368	60.81	7.96	34.45	12.19
1	M	56	140	46.34	18.22	132.42	9.58
1	F	54	44	122.11	38.65	124.11	3.09
1	M	44	116	112.01	13.88	58.73	4.14
1	M	53	76	7.84	1.30	2.08	4.77
1	M	67	56	37.04	2.73	20.31	3.80
1	F	63	48	4.66	1.83	4.42	2.56
1	M	64	12	131.89	23.74	162.41	4.88
1	M	74	4	11.60	4.51	6.65	4.74
1	M	45		18.56	3.52	1.84	3.38
1	F	35	96	23.84	8.42	8.85	2.32
1	F	78	164	104.67	13.23	50.36	11.77
1	M	59	108	11.62	11.49	3.27	7.39
1	M	64		61.89	15.46	30.62	14.80
1	F	75	12	13.49	6.74	8.97	8.98
1	M	57	56	377.85	76.99	146.46	21.55
1	M	47	72	72.93	8.75	23.56	5.42
2	M	22	60	2.13	5.70	11.05	3.64
2	F	15	24	22.86	7.51	16.91	1.87
2	M	40	40	3.78	3.38	18.97	5.83
2	F	17	44	0.81	18.89	20.50	2.32
2	F	33	48	1.35	0.76	1.65	1.02
2	M	28	12	0.00	1.84	5.87	1.53
2	F	18	4	14.08	1.93	15.38	2.77
2	M	17	32	5.40	1.29	5.53	1.99
2	F	17	28	0.00	0.38	5.26	0.91
2	F	24	136	2.95	1.17	7.52	1.84
2	M	40	68	1.55	2.85	6.76	2.25
2	F	36	32	0.00	0.00	2.73	3.00
2	M	40	4	14.05	2.54	39.30	3.66
2	M	24	48	3.57	0.75	3.05	2.50
2	F	28	16	3.68	0.68	7.91	3.09

No.1 means OSCC and No.2 means normal control

Table 3. The means and standard deviations of expression levels of five factors and the P values of t-tests

	Angiogenin	TGF-β	VEGF	APEX	TNF-α
OSCC					
mean	98.67	48.48	9.20	30.96	4.34
standard deviation	121.93	61.64	13.21	40.05	4.04
Normal					
mean	39.73	8.62	3.31	14.08	2.55
standard deviation	32.76	6.65	4.78	9.84	1.23
P value	0.0037	< 0.0001	0.0102	0.0023	0.0074

Table 4. The sensitivity and specificity for cut-off value analysis

	angiogenin	TGF-β	VEGF	APEX	TNF-α
cut-off value	68.95	26.8	6.5	21.4	3.4
sensitivity(%)	41	46	44	41	40
specificity(%)	82	100	78	89	83
cut-off value	59.1	18.55	4.55	14.98	2.38
sensitivity(%)	46	60	52	47	58
specificity(%)	82	90	72	61	61
cut-off value	49.1	15.9	3.9	12.84	2.04
sensitivity(%)	51	65	54	49	79
specificity(%)	71	89	72	61	44
cut-off value	40.25	13.25	3.25	10.7	1.7
sensitivity(%)	58	69	60	49	85
specificity(%)	60	78	67	50	22

Table 5. Pearson's coefficeints and P values for linear regression analysis of five factors for cancer patients

	angiogenin	TGF-β	VEGF	APEX	TNF-α
angiogenin					
Pearson's coefficeint		-0.11040	-0.01600	0.02833	0.29255
P value		0.4501	0.9131	0.8484	0.0392
TGF-β					
Pearson's coefficeint	-0.11040		0.80729	0.71836	0.57146
P value	0.4501		<0.0001	< 0.0001	< 0.0001
VEGF					
Pearson's coefficeint	-0.01600	0.80729		0.74377	0.58828
P value	0.9131	< 0.0001		< 0.0001	< 0.0001
APEX					
Pearson's coefficeint	0.02833	0.71836	0.74377		0.49755
P value	0.8484	< 0.0001	< 0.0001		0.0002
TNF-α					
Pearson's coefficeint	0.29255	0.57146	0.58828	0.49755	
P value	0.0392	< 0.0001	< 0.0001	0.0002	

precursors, shed from the vessel wall or mobilized from the bone marrow, also contribute to tumor angiogenesis[33]. Contrarily, adult normal tissue vasculature is quiescent because of the balance between angiogenic promotors and inhibitors. The early development of tumor is dormant because of the same reason. Break of the balance results in physiological angiogenesis such as wound healing and pathological angiogenesis such as tumor angiogenesis. Tumor-induced angiogenesis is thought to depend on the production of pro-angiogenic growth factors by the tumor cells, which affect the existing vessels[2]. This turns on the tumor "angiogenic switch", and then promotes tumor growth and metastases.

Angiogenin may serve multiple roles in cancer progression through different processes, such as angiogenesis and adhesion and then invasion; however, the critical role of angiogenin in cancer progression is still controversial. According to our result, the expression of angiogenin in OSCCs was significantly higher than normal tissues ($P<0.0037$), so we could know that angiogenin might serve important toles in OSCC progression and OSCC might be a highly vascularized cancer.

TGF-β has an ambivalent activities in tumor angiogenesis. TGF-β has been shown to regulate new blood vessel formation both *in vitro* and *in vivo* by a combination of responses that include increased production and facilitation of VEGF, facilitation of basic fibroblast growth factor mediated capillary sprouting, inhibition of endothelial cell migration, and increased production of extracellular matrix, among others[25]. In our study, the expression levels of TGF-β in OSCCs were much higher than normal ($P<0.0001$). It was also similar to other reports[28,29].

VEGF, recognized as the most important angiogenic factor, is a multifunctional cytokine that is widely expressed by tumor cells and increases microvascular permeability, induces endothelial cell migration and division, and promotes endothelial cell survival[13]. It may also act as an autocrine survival factor for some cancer cells[35]. There are some works that the expression of VEGF could predict the metastasis of oral cancers[36-38]. The results are controversial. Some studies strongly imply that the expression of VEGF correlates with the metastasis[36,37], whereas the others shows no correlation[38]. Although further investigations are needed to find the value of VEGF to predict the metastasis of oral cancers, most investigators are agreed to accept the expression of VEGF as a diagnostic marker for cancer detection. Like other many studies, we showed that the expressions of VEGF in oral cancers are higher than normal tissues ($P<0.0102$).

APEX is the major human endonuclease that plays a key role in the DNA base excision repair process. We analyzed the expression of APEX and showed close correlation between the expression of APEX and OSCC ($P<0.0023$). As we know, the importaqnt role of APEX is the DNA base excision in repair process. How we can explain the higher expression of APEX in OSCC tissues than normal control tissues. It might be that more active repair processes following the destructive processes in OSCC tissues than normal oral tissues are needed.

TNF-α has a wide range of biological activities, affecting the growth, differentiations, or function. Although the complexity of TNF-α activities makes it at present difficult to assign a specific role to TNF-α *in vivo*, its direct tumoricidal activity and its role in inflammation are thought to contribute to the host defense against tumors. This proposal has been challenged by findings indicating that many cell types, including tumor cells themselves, are able to produce either TNF-α or lymphotoxin[39]. Lymphotoxin is a TNF-α related cytokine that binds to the same receptor and exerts similar biological activities. TNF-α or lymphotoxin production by tumor cells suggests that these cytokines have a possible pathogenic role in tumorigenesis. In addition, because of its potent angiogenic activity[40], TNF-α may stimulate the growth of blood vessels, thereby promoting tumor development. Furthermore, the ability of TNF-α to induce collagenase synthesis may lead to tissue destruction facilitating the invasive growth of tumors. Thus, demonstration of TNF-α production in cancer may reflect either protective or pathogenic processes. Despite of its controversial role of tumorinogenetic and tumoricidal activity, the expression levels of TNF-α in oral cancers are observed much higher than normal tissues[41,42]. Our results of TNF-α expressions in OSCCs were much higher than normal controls ($P<0.0074$).

We got results that the expressions of angiogenin, TGF-β, VEGF, APEX, and TNF-α in OSCCs were much higher than normal contols. These were similar to other studies. We analyzed statistically to find the cut-off values that would be used as diagnostic markers between cancer and normal tissues, but could not find that. It means that there are significant differences between cancer and normal tissues, but until now the expression levels of these five factors are unuseful for the diagnosis to detect OSCC. Also we took the linear regression analysis

between every two factors of these five factors. There were no significant correlations between angiogenin and the others except angiogenin and TNF-α. The rest four factors except angiogenin showed close correlation between any combination of them. It is uncertain why there were no correlations between angiogenin and the others and there were close correlations between any combination of the others. Our results suggest that not only angiogenin, TGF-β, VEGF, APEX and TNF-α are significantly associated with the tumorigenesis, but also the close relationship between these factors might enhance the tumorigenesis of OSCC.

From these findings, we could say that all of these five factors had certain effects on tumorigenesis of OSCC and except angiogenin the rest four factors had close correlations between each other. The exact nature of these five factors on tumorigenesis in OSCC is still being investigated. In general, oral cancers are easily detected in routine intra-oral examinations. So further studies are reguired that the expression levels of these five factors can predict the metastasis of OSCC, stage of OSCC, and finally estimate the prognosis of OSCC.

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