Ataxia-telangiectasia-mutated protein expression as a prognostic marker in adenoid cystic carcinoma of salivary glands

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Directed by Professor Jin Kim

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Shadavlonjid Bazarsad

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The Graduate School
Yonsei University

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(Directed by Professor Jin Kim)

Adenoid cystic carcinoma (ACC) is one of the high grade malignant tumors in salivary glands which is characterized by multiple recurrences and late distant metastasis. Recently, Myb-NFIB fusion or rearrangements of Myb have been detected as a hallmark of ACC. However, no biological marker estimating the outcome of ACC has been proven yet. The serine/threonine protein kinase ataxia telangiectasia mutated (ATM) is critical in maintaining genomic integrity. The encoded protein plays a central role in the complex processes that repair DNA double strand breaks. ATM expression in salivary glands tumor has not been reported previously. Purpose of this study was to determine the ATM gene instability and the status of its protein expression in carcinoma or cancer associated stroma of ACC of the salivary glands related to its survival. ***Experimental Design:*** This study consists of 2 different experimental parts dependent on research purpose. The first part consists of 11 surgical ACC samples for searching loss of heterozygosity (LOH). For this study, the primers for 7 sites in chromosome 9, for 2 sites in chromosome 11 and for 4 sites in chromosome 21 were applied for the detection of LOH. The second part consists of 48 surgical samples for detecting expression of ATM and its downstream p53 and for investigating their prognostic effects. Kaplan-Meier plots were used to evaluate the relationship between the protein

expression ratios of ATM, p53 and its ATM-mediated phosphorylation and the overall survival rate of patients with ACC. ***Results:*** The highest frequency of LOH in both cancer cells and stromal fibroblasts in ACC was found in D11S1778 region. LOH of D11S1778 was found in 4 cases of 11 patients in cancer cells and 5 cases in stromal fibroblasts of ACC. One of the candidate genes located in this region is *ATM*. Therefore, the further study focused on searching the prognostic effects of ATM and its downstream protein expression in ACC. As results, low expression of ATM in cancer cells correlated with poor survival rate ($p = 0.037$). However, low expression of ATM in stromal fibroblasts was not significantly associated with patient outcome. Moreover, this study evaluated ATM expression stratified by p53 and its ATM-mediated phosphorylation status. ATM loss was associated with a significantly decreased overall survival in patients simultaneously showing overexprssion of p53 ($p = 0.01$) and low expression of p53 phospho S15 ($p = 0.05$). These data supported that loss of ATM and its functional status in p53 pathway is an important factor associated with poor outcome of patients in ACC of salivary glands.

Keywords; Adenoid cystic carcinoma, *ATM* gene, LOH, protein expression, p53, p53 phospho S15

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I. INTRODUCTION

Adenoid cystic carcinoma (ACC) is a locally aggressive salivary gland neoplasm which is characterized by its long clinical history with multiple recurrences and late distant metastasis (Nagao et al., 2003). Distant metastasis can develop in many patients more than 10 years after initial treatment. Ten-year survival has been observed to decrease by 30% in patients with ACC who develop distant metastasis. One report noted that 47% of patients with early-stage ACC developed distant metastasis (Bhayani et al., 2012). Therefore, understanding molecular mechanism influencing prognosis are critical to improve the outcomes in patients with ACC.

One of the main oncogenic drivers of ACC is the tumor-type–specific chromosomal translocation t(6;9) (q22-23;p23-24) resulting in an *Myb-NFIB* fusion oncogene. *Myb-NFIB* fusion contributes to Myb overexpression and to the activation of critical Myb targets, including genes associated with apoptosis, cell cycle control, cell growth/angiogenesis, and cell adhesion (Bell et al., 2011). Recent studies in the surgical pathology literature have revealed that overexpression of Myb occurs in most ACCs (89

%), including ACCs without the *Myb-NFIB* fusion, suggesting that Myb expression may be useful as an ancillary marker for this tumor (Brill et al., 2011; West et al., 2011). *Myb-NFIB* fusion or rearrangements of Myb have not been detected in any other subtype of salivary gland cancer, indicating that Myb activation is a hallmark of ACC (North et al., 2015). Even the importance as a diagnostic marker of Myb activation in ACC, Myb expression showed no significant effect on patient's survival (Bell et al., 2011).

Ataxia telangiectasia mutated (ATM) is a 370-kd protein belonging to a family of PI-3 protein kinases, with a role in DNA processing, regulation of the cell cycle, and control of telomere length. The principal function of the ATM is the integration of cellular responses to DNA double-strand breaks (Shiloh, 2001). The homozygous mutation of the *ATM* gene results in the loss of detectable protein which cause ataxia-telangiectasia (A-T) (Zhao et al., 2011), an autosomal recessive disorder characterized by neurological and immunological symptoms, radiosensitivity and predisposition to cancer, particularly of the lymphoid system (Gilad et al., 1996; Guarini et al., 2012). Accumulating evidence provides that A-T families have shown A-T heterozygotes, also have an increased risk of developing cancer, in particular breast cancer, for which female ATM carriers have increased risk compared with the general population (Janin et al., 1999). Moreover, the loss of ATM expression and poor prognosis has been reported in pancreatic cancer and gastric cancer (H. Kim et al., 2014; J. W. Kim et al., 2014). Recently one report has demonstrated that ATM expression in malignant tumor as well as cancer associated stroma is an independent prognostic marker in early stage of breast cancer (Feng et al., 2015). As our understanding that cancer development extends beyond single gene disorders to multigenetic disorders, it is tempting to speculate that the combined effect of several defective genes in a common antitumour pathway could lead to more poorly differentiated tumours. The frequency of the loss of ATM protein has not been reported in ACC of salivary glands previously. Since there is no biological marker to estimate the outcome of ACC patients in salivary glands, this study attempted to investigate the relationship between expression pattern of ATM and p53 and patient survival in ACC patients. These data would contribute to predicting prognosis and improving survival in ACC patients.

II. MATERIALS AND METHODS

1. Human tissue samples

This study consisted of 2 different parts of population dependent on research purpose. The first part consisted of 11 surgical samples for searching LOH. The second part consisted of 48 surgical samples for immunohistochemical (IHC) study. This study has been approved by an ethical committee from the Institutional Research Board of the Faculty of Dental Sciences, Yonsei University, South Korea (No: 2-2015-0031). All samples were diagnosed as ACC of salivary gland at Collage of Dentistry, Yonsei University from 1998-2015. Patient's clinical data were collected retrospectively through chart review. All cases were confirmed histologically in accordance with the latest World Health Organization classification of salivary gland tumours, based on hematoxylin and eosin stained tissue sections. There were no age, sex, ethnicity, or tumor stage restrictions on patient enrollment. Follow-up was calculated as the time from the first appointment at the institution for the primary tumour to the date of death.

2. Laser Capture Microdissection (LCM)

LCM was performed on 11 macro-dissected surgical samples. Formalin fixed paraffin embedded tissue was cut into 10 serial sections with a thickness of 10 μm, and sections were mounted on Arcturus® membrane glass slides and stored at – 80°C. Before LCM, sections were deparaffinized through a series of xylene baths and rehydrated followed by stepwise dehydration with 100%, 95%, 75% ethanol for 1 minute each. Tissue sections were washed by RNase-free water for 30 seconds. Next, the sections were counterstained with Mayer's hematoxylin followed by sterilized distilled water washing and 0.1% sodium bicarbonate for 30 seconds. Tissue sections were placed in 70% ethanol for 30 seconds followed by eosin staining. Finally tissue sections were dehydrated by series of alcohol for 30 seconds each, and xylenes for 2 minutes. After air drying of the sections, target cancer cell nests and cancer associated fibroblasts were respectively delineated with markers on the monitor and selectively microdissected from the section according to the standard procedure using a PixCell II LCM system (Arcturus Engineering, Mountain View, CA/Olympus, Tokyo, Japan).

3. DNA extraction

The commercial QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. The tissues were dewaxed with xylene, followed by two washes with 100% ethanol twice to remove residual xylene. After dewaxing, tissues were digested overnight at 56°C with 180µl of ATL buffer and 20µl proteinase K. After digestion, 200 µl of AL buffer was added and incubated at 70°C for 10 minutes, followed by ethanol precipitation. The solution was transferred into a spin column, and washed with washing buffers provided in the kit. DNA was eluted with 100µl of AE buffer. The quality (OD260/OD280) and quantity (OD260) of DNA was measured spectrophotometrically using the standard method.

4. PCR Amplification

Extracted DNA preparations with heat treatment method were subjected to DNA amplification. Chromosome 9, 11, 21 primer sequences are summarized in Table 1. Amplification was set up in a 20 ml reaction master mixture containing 1 µM of primers and 1µg of genomic templates. The PCR conditions were as follows: Initial denaturation at 95ºC for 3 minutes, followed by 35 cycles of amplification at 94ºC for 30 seconds, annealing for 1 minute at the respective annealing temperature and extension at 72ºC for 1 minute followed by an additional extension for 5 minutes at 72ºC. Twenty microliter of PCR products were electrophoresed on a 1.5% agarose gel.

Table 1. Primers sequences for PCR

	Locus	Sequences
9p	D9S162	GCA ATG ACC AGT TAA GGT TC
		AAT TCC CAC AAC AAA TCT CC
	D9S168	GGT TTG TGG TCT TTG TAA GG
		TGG TTT GTT TGT ATA ACT ATC ATT G
	D9S171	AAG TGA ACC TCA TCT CTG TCT G
		CAA CCC TAG CAC TGA TGG TA
	D9S286	TGC ACT TGT ACA CAT ATG CTG
		CTT TGC CTG CAT GCT CAC
	D9S177	CAG GGG TCA GAA TCT TAA AG
		CCC TTC ATA CAA AAA CTA CCA C
	D9S1748	CAC CTC AGA AGT CAG TGA GT
		GTG CTT GAA ATA CAC CTT TCC
	D9S1749	AGG AGA GGG TAC GCT TGC AA
		TAC AGG GTG CGG GTG CAG ATA A
11q	D11S4108	TGG CAA GTG GCA GGA T
		GCC CAT AGA TGG ATG AGT AGA
	D11S1778	TAA CCT NCT ACA CAG TGT CGT T
		GCT TCA GCA GAG AAG CCA T
21q	D21S1904	ATG AGT TCA GTG TTT CAT GGA CAT C
		AGC AAG ATT ACT GTC TGG TTT CCC
	D21S120	GTG TGT CTG CCA TTT CTG GGT GTA G
		GAT CCT GGG ACA AAG TAG TCT CTA A
	D21S1911	AGC TCC TGA GGA GAC ATC C
		TCC CTT ACA TAC ACA CAG CA
	D21S1922	ATG CTA TCT TGT CAA AAT ATG TG
		TGG AAA TAT ATA AAC AAA TCA CTG G

5. LOH Analysis

To determine LOH or microsatellite instability (MSI), the visual signal intensity was evaluated by densitometry. LOH was scored when the band intensity of an allele in tumor DNA showed a complete loss or more than 50% reduction in intensity compared with that of the corresponding normal allele. MSI was recorded when an expansion, contraction, or an additional allele was found in the tumor sample in comparison to matching normal mucosa.

6. Hematoxylin and Eosin Staining

Formalin-fixed paraffin-embedded specimens were cut into 4-μm-thick sections and were stained with hematoxylin and eosin for histologic confirmation of clinical diagnosis and to select appropriate tissue area. Additional sequential sections were prepared for immunohistochemical studies.

7. IHC Protocol

Forty-eight cases of ACC were available for high-quality IHC staining. The staining was performed on 4-µm-thick sections using an EnVision-HRP detection system (Dako, Carpinteria, CA). All of the procedures were performed at room temperature. Sections were deparaffinized through a series of xylene baths and rehydrated in graded concentrations of alcohol. To retrieve antigenicity, the slides were steamed with 10 mmol/L citrate buffer (pH 6.0; DakoREAL^{TM}). Tissue sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity, followed by incubation in 5% bovine serum albumin. Sections were then incubated with primary antibodies diluted with phosphate-buffered saline for 90 minutes in a humid chamber. Positive and negative controls comprising appropriate tissue and omission of the primary antibody, respectively, were included. Primary antibodies from Abcam were used anti-Myb rabbit monoclonal antibody (clone EP769Y); 1:100 dilution ratio, ATM a rabbit monoclonal antibody (ab32420); 1:50 dilution ratio, anti-p53 phospho S15 (ab38497); 1:100 dilution ratio, p53 (FL-393) rabbit polyclonal antibody (sc-6243) from Santa Cruz; 1:50 dilution ratio respectively (Table 2). The slides were then incubated with secondary EnVision reagent (Dako) for 30 minutes, followed by incubation with diaminobenzidine chromogen. Next, the sections were counterstained with Mayer's hematoxylin for visualization. The phosphate-

buffered saline solution without primary antibody treatment was applied as the negative control for all antibody staining. Positive controls consisted of human lymphocytes and normal salivary glands used for ATM staining as recommended by Villaruz LC et al (Villaruz et al., 2016). Paraffin embedded breast cancer cell line was used as to determine appropriate dilution ratio and positive control for p53 phospho S15. The percentage of positive cells was used, with a cut off of ≥ 25% cells being evaluated as positive nuclear Myb (North et al., 2015), whereas "Loss of ATM expression" was defined as tumours with more than 25% of ATM negative tumour cells as showing reduced expression of these proteins (Abdel-Fatah et al., 2008). Since mutant TP53 proteins generally have a longer half-life than wild-type TP53 protein, which leads to their nuclear accumulation, tumours with more than 20% of nuclear TP53-positive tumour cells were classed as showing aberrant expression (Abdel-Fatah et al., 2010).

8. Statistical analysis

Correlation between clinical findings of patients and cancer risk were analyzed by Fisher's exact test. Cumulative survival probabilities were estimated using the Kaplan–Meier method. All of the statistical analyses were performed using statistical software (SPSS ver. 21.0, SPSS Inc. Chicago, IL, USA), and the level of statistical significance was set at $p<0.05$.

Table 2. Antibody informations

Antibody characteristics

	Biomarkers	*Company*	*Catalog No.*	*Dilution*	Scoring system	Cut-off	Reference
1	*Myb*	Abcam	ab45150	1:100	Positive cells (%)	≥25% positive	North Jp et al, 2015
2	ATM	Abcam	ab16667	1:100	Positive cells (%)	<25% negative	Yoshikawa et al, 1999; Ange`le et al, 2000
3	p53	Santa cruiz	sc-6243	1:50	Positive cells (%)	>20% positive	Finlay et al, 1988
4	p53 S15	Abcam	ab38497	1:100	Positive cells (%)	>20% positive	

III. RESULTS

#Part 1

1. Detection of LOH in ACC

The first part of our study aimed to detect LOH region which would be frequently affected in ACC of salivary glands. Eleven ACC patients samples were analyzed LOH in DNA from cancer cells and cancer associated fibroblasts, respectively. The age range was 44–68 years; median age at diagnosis was 58 years. Among the 11 patients, 6 (54.5%) were female, 5 (45.5%) were male whereas less than 58 years patients were 6 (54.5%) and more than 58 years patients were 5 (45.5%) (Table 3). DNA from cancer cells and stromal fibroblasts were analyzed for LOH with three microsatellite markers which are located at chromosome subband 21q, 11q and 9p. The frequencies of LOH of different markers are summarized in Table 4 and 5. The marker that showed the highest frequency of LOH in both cancer cells and stromal fibroblasts in ACC was D11S1778. LOH of D11S1778 was found 4 of 11 cases in cancer cells and 5 of 10 in stromal fibroblasts of ACC, whereas 2 cases were found noninformative. Two patients were found MSI and LOH simultaneously in cancer cells and stromal fibroblasts. From these data, *ATM* gene was selected as one of the candidate genes located in this region. These data led us to further study to check ATM protein expression in ACC patients.

Table 3. Baseline characteristics of Part 1.

Parameter		No. of Patients n(%)
Sex		
	Female	6(54.5)
	Male	5(45.5)
Age (years)		
	< 58	6(54.5)
	≥ 58	5(45.5)
Tumor site		
	Parotid gland	3(27.2)
	Submandibular gland	6(54.5)
	Palate	1(9.09)
	Floor of mouth	1(9.09)
Histology	Solid	2(18.2)
	Tubular and cribriform	9(81.8)
All patients		11(100)

Table 3. LOH of cancer cells in Adenoid cystic carcinoma

locus		1	2	3	4	5	6	7	8	9	10	11
21q	D21S1904	NI	NI	□	NI	□	NI	LOH	□	□	□	NI
	D21S120	NI	NI	□	NI	□	□	NI	NI	NI	NI	NI
9p	D9S168	□	NI	NI	□	LOH	□	LOH	□	NI	□	□
11q	D11S1778	□	LOH	□	MSI	LOH	□	LOH	□	NI	NI	NI

Table 4. LOH of stromal fibroblasts in Adenoid cystic carcinoma

		1	2	3	4	5	6	7	8	9	10	11
21q	D21S1904	NI	NI	LOH	NI	□	NI	□	□	□	□	NI
	D21S120	NI	NI	□	□	□	□	NI	NI	NI	NI	NI
9p	D9S168	NI	NI	NI	□	LOH	LOH	□	□	□	□	□
11q	D11S1778	MSI	□	LOH	MSI	LOH	LOH	□	□	□	NI	NI

#Part 2. Detection of prognostic value of ATM protein status in ACC

1. Characteristics of the patients

The second part of our study was to examine ATM protein expression in ACC. The clinico-pathological characteristics of the patients are listed in Table 6. There are 48 patients were enrolled in this study from July 1990 to October 2015. The age range was 26–71 years; median age at diagnosis was 55 years. Among the 48 patients, 23 (47.9%) were female, 25 (52.1%) were male, whereas less than 55 years patients were 26 (54.1%) and more than 55 years patients were 22 (45.8). Thirty six cases (75%) were found in minor salivary glands and majority of cases 23 (48%) were in palate followed by the submandibular gland 8 (16.7%), floor of mouth 6 (12.5%) and parotid glands 3 (6.3%). Histologically 13 (27.1%) cases were diagnosed as ACC solid type, 35 (72.9%) cases were diagnosed as ACC tubular and cribriform type. 36 (75%) cases were found with perineural invasion. The medium follow up period for the patient population was 5 years. Five patients (10.4%) among 48 patients died during follow up period. Among 5 dead patients, 4 patients (80%) were found to be more than 55 years, all tumors arose in minor salivary glands and 2 of them were in solid type and 3 of them were found with perineural invasion. Since histological hallmark of poor prognosis are histological type and perineural invasion, Kaplan-Meiyer analysis was conducted whether these histologic findings affected on their prognosis. But because of small number of patient, histologcal type was not significant in Kaplan-Meiyer analysis. In case of perineural invasion, it was not statistically significant (p=0.844) (Figure 2).

2. Myb expression pattern in ACC

All 48 ACC patients sample were screened for Myb staining. Myb expression was not detected in non-tumoral salivary gland parenchyma. Overall, 41 of 48 (82%) ACC were stained positively for Myb. Myb expression was entirely nuclear, and ranged from negative to strongly positive. In tubular foci, staining was predominantly found in myoepithelial cells and lack of expression in ductal cells (Figure 1). There was no statistically significant difference in Myb staining according to anatomic site of the tumors, or clinical features of the patients (Table 6). Note of that Myb expression was found significantly related to histological type. Thirty three of 35 (94.3%) tubular and cribriform type of ACC were found to be positive for Myb, whereas 8 of 13 (61.5%) solid type of ACC were found to be positive for Myb. These difference was statistically significant (p=0.011). However, Myb expression difference according to perineural invasion which is well known as a histological prognostic marker showed no significance. Moreover, Myb expression showed no statistical significance in predicting ACC patient outcomes (Figure 2).

Table 6. Baseline characteristics of patients in Part 2.

Parameter		No. of Patients	Myb positivity		p value
			n	%	
Sex					0.175
	Female	23(47.9)	18	78.3	
	Male	25(52.1)	23	92.0	
Age (years)					0.403
	< 55	26(54.1)	23	88.5	
	≥ 55	22(45.8)	14	81.8	
Tumor site					0.523
	Parotid gland	3(6.3)	3	100	
	Submandibular gland	8(16.7)	5	62.5	
	Sublingual gland	1(2.1)	1	100	
	Palate	23(48)	17	74.0	
	Upper lip	2(4.2)	1	50.0	
	Lower lip	1(2.1)	1	100	
	Tongue	2(4.2)	1	100	
	Buccal	1(2.1)	0	0	
	Retromolar area	1(2.1)	0	0	
	Floor of mouth	6(12.5)	5	83.3	
Histology					**0.011**
	Solid	13(27.1)	8	61.5	
	Tubular and cribriform	35(72.9)	33	94.3	
Perineural invasion		36(75.0)	21	58.8	0.739
All patients		48(100)	41	82.0	

****P values from Fisher's exact test are provided*

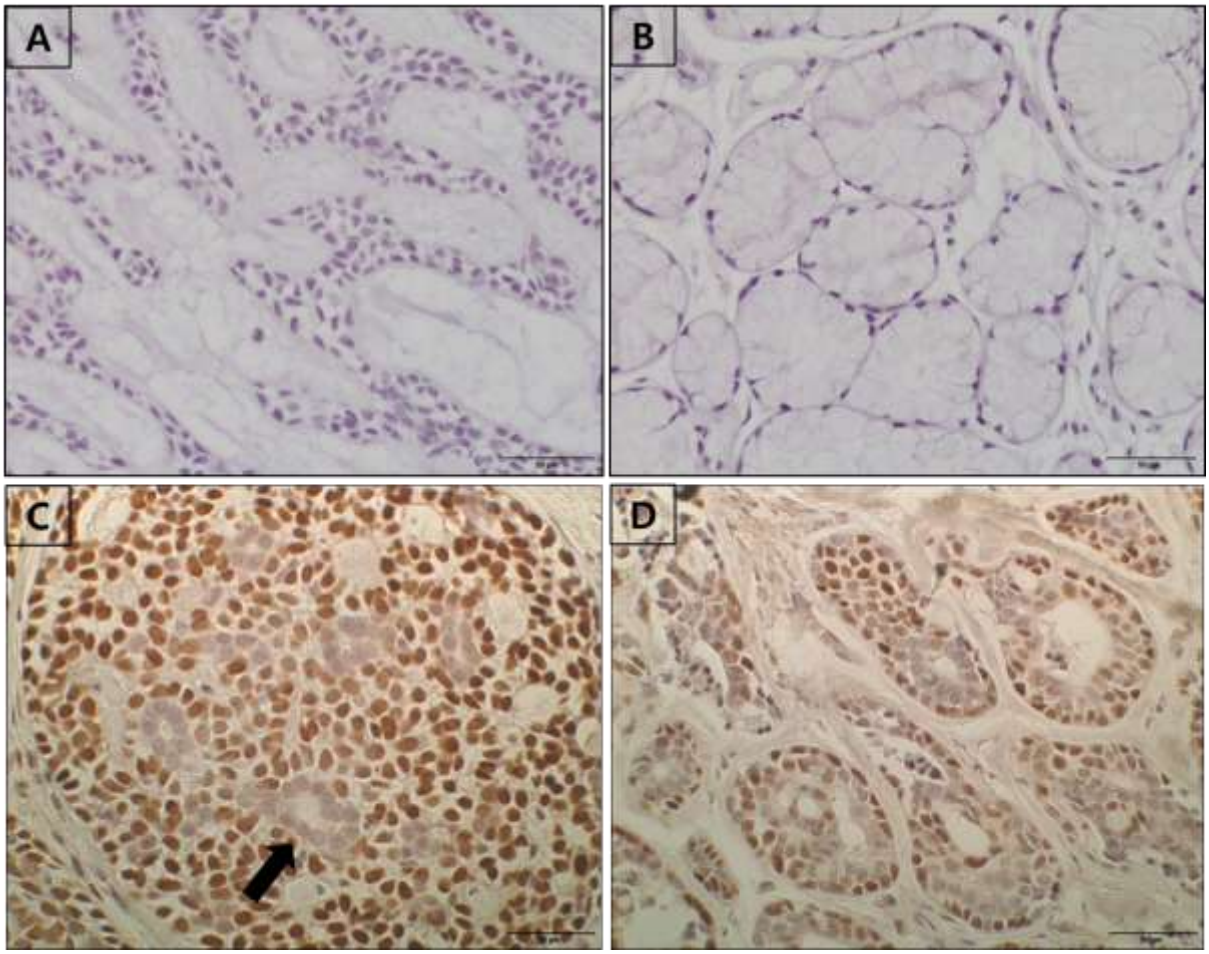

Figure 1. IHC expression of Myb in ACC (A) Negative control by omission of the Myb antibody in ACC, (B) Myb expression was not detected in non-tumoral salivary gland parenchyma, (C) Myb expression in ACC solid type. Myb staining is often restricted myoepithelial cells and lack of expression in ductal cells, indicated by the black arrow, (D) Myb expression in ACC tubular and cribriform type. The original magnification of all figures was taken at ×400.

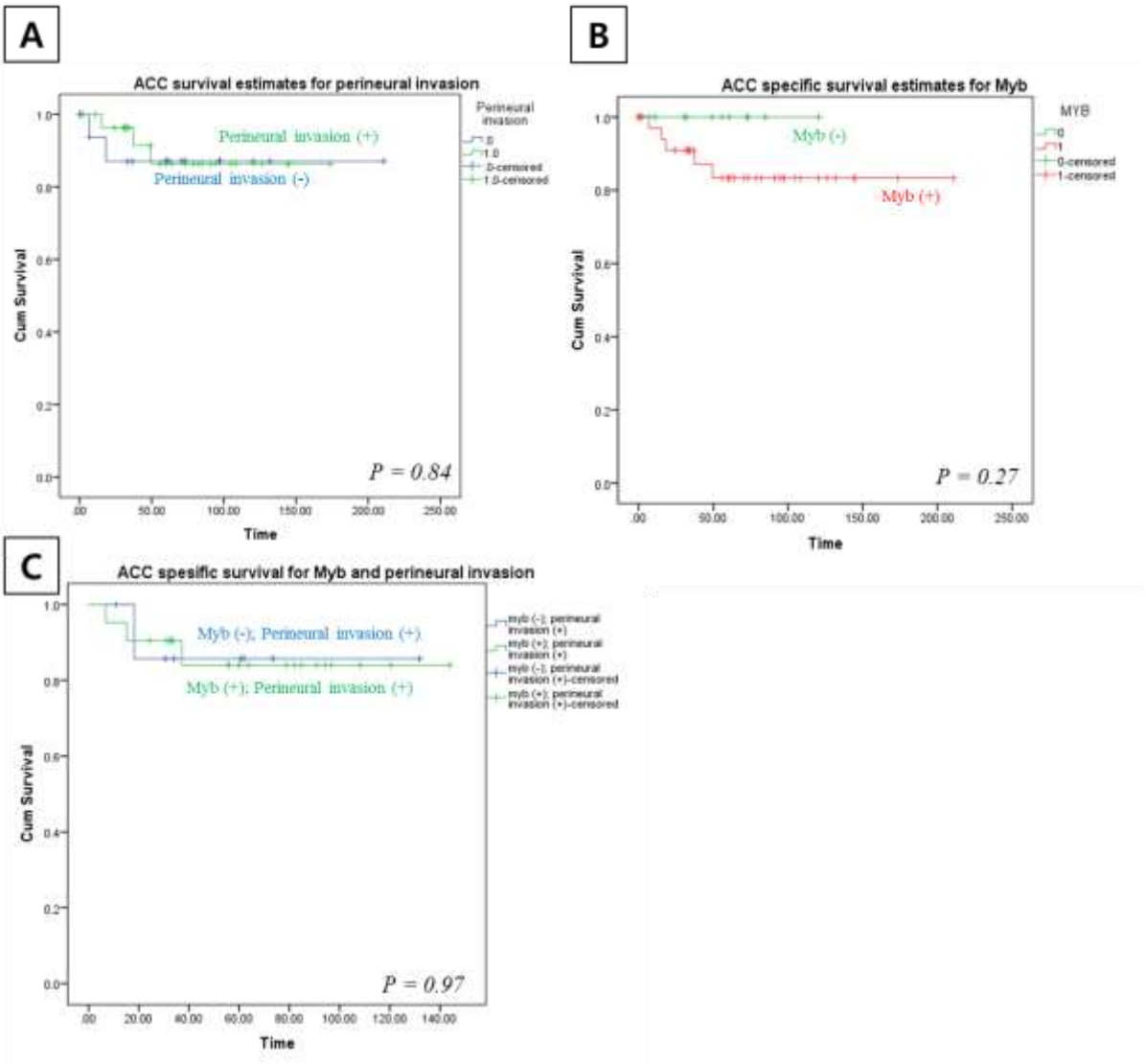

Figure 2. Overall survival according to (A) perineural invasion, (B) Myb expression in ACC (C) Myb expression difference according to perineural invasion.

3. Relationship between ATM protein level in ACC and patient outcome

ATM protein expression level in 48 ACC study population was assessed. Antibody specificity was validated using human lymph node tissue as positive control. For negative control, entire procedure was conducted except omission of ATM antibody (Figure 3A and B). ATM protein was detected in the nucleus of the ducts and acini in the normal salivary glands. ATM protein was found negative to moderate positive nuclear expression in both cancer cell and stromal fibroblasts in ACC (Figure 3). Aberrantly reduced/absent expression of ATM was found in 58.3% of patients in cancer cells and 60.4% of patients in stromal fibroblasts compared to adjacent normal tissue. ATM protein expression in cancer cells was significantly lower in older patients compared to younger patients ($p = 0.003$). Moreover 75.9% of patients who have loss of ATM in stromal fibroblasts was found significantly related to perineural invasion ($p=0.008$) in Table 7. The Kaplan–Meier analysis revealed that the low ATM expression in cancer cells was correlated with poor survival. On the other hands, the low ATM expression in stromal fibroblasts was not significantly associated with patient outcome (Figure 4).

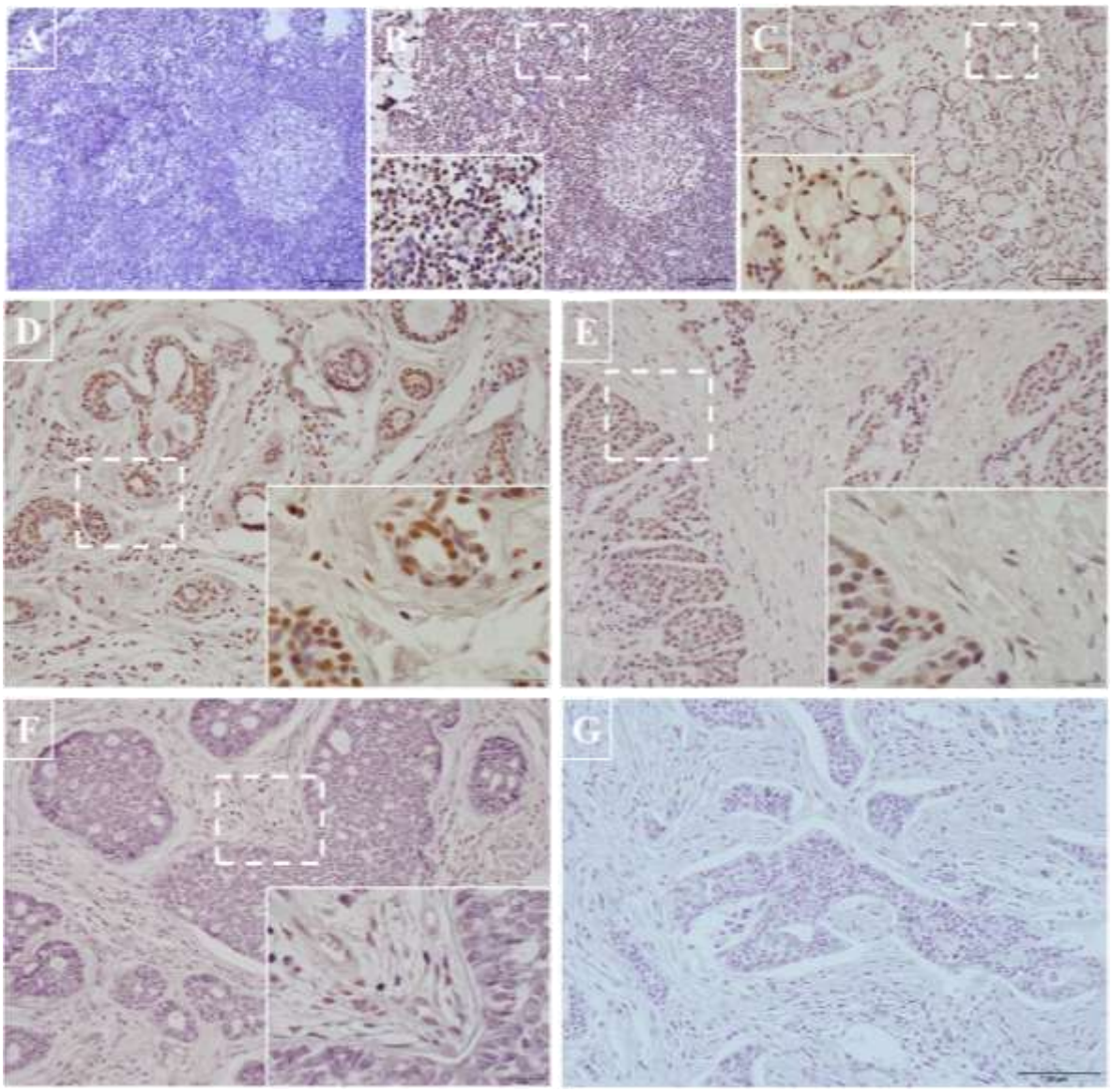

Figure 3. IHC expression of ATM in ACC. (A) Negative control by omission of the ATM antibody (B) Positive control. ATM antibody specificity staining in human lymph node (C) nuclear ATM expression is seen in most nuclei of ducts and acini in the normal salivary gland. (D) ATM staining positive in cancer cells and stromal fibroblasts. (E) ATM cancer positive, stromal fibroblast negative. (F) ATM cancer negative, stromal fibroblast positive (G) ATM negative both cancer and stromal fibroblasts .The original magnification of all figures was taken at ×200. Higher magnification (x1000) views

Table 7. Relationship between ATM, p53 and p53 S15 phospho proteins in ACC and patient outcome

	Parameter	No. of Patients	Loss of ATM in cancer		Loss of ATM in stroma		Overexpression of p53		Overexpression p53 phospho S15	
		n(%)	n	%			n	%	n	%
Sex										
	Female	23(47.9)	15	65.2	16	69.6	16	69.6	9	31.9
	Male	25(52.1)	13	52.0	13	52.0	22	88.0	9	36.0
	p value			0.263		0.172		0.112		0.529
Age (years)										
	< 55	26(54.1)	10	38.5	14	53.8	21	80.8	9	34.6
	≥ 55	22(45.8)	18	81.8	15	68.2	17	77.3	9	10.9
	p value			**0.003**		0.238		0.521		0.440
Tumor site										
	Parotid gland	3(6.3)	1	33.3	2	66.6	3	100	0	0
	Submandibular gland	8(16.7)	4	50.0	5	62.5	6	75.0	6	75.0
	Sublingual gland	1(2.1)	1	100	1	100	1	100	1	100
	Palate	23(48)	14	60.8	15	65.2	17	73.9	8	34.7
	Upper lip	2(4.2)	1	50.0	1	50.0	2	100	1	50.0
	Lower lip	1(2.1)	0	0	0	0	1	100	0	0
	Tongue	2(4.2)	2	100	1	50.0	2	100	0	0
	Buccal	1(2.1)	0	0	0	0	1	100	0	0
	Retromolar area	1(2.1)	0	0	0	0	1	100	0	0
	Floor of mouth	6(12.5)	5	83.3	4	66.6	4	66.6	2	33.3
	p value			0.475		0.863		0.992		0.219
Histology										
	Solid	13(27.1)	10	76.9	8	61.5	7	53.8	4	30.8
	Tubular and cribriform	35(72.9)	18	51.4	21	60	31	88.6	14	40
	p value			0.102		0.597		**0.016**		0.740
Perineural invasion		36(75)	17	60.7	22	75.9	23	60.5	11	61.1
				0.597		**0.008**		0.624		0.592
All patients		48(100)	28	58.3	29	60.4	38	79.0	18	38.0

****P values from Fisher's exact test are provided*

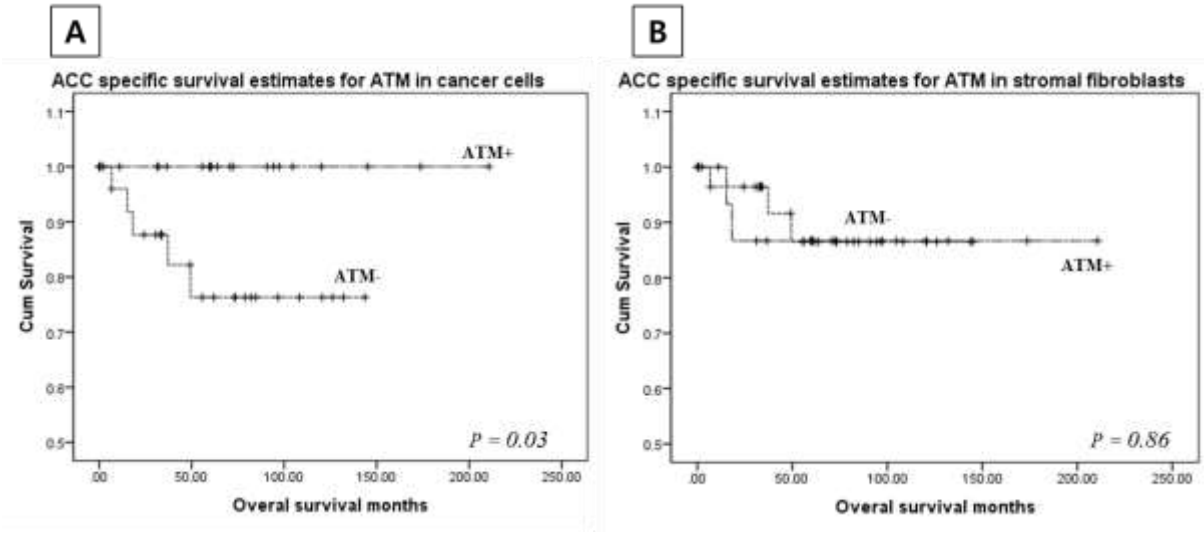

Figure 4. Overall survival according to ATM protein expression in (A) cancer and (B) stromal fibroblasts.

4. p53, p53 phospho S15 proteins level in ACC and their coexpression with ATM and patients outcome

Of the 48 patients, 38 (79.1%) of patients for p53 and 30 (62.5%) of patients for p53 phospho S15 were found overexpression (Figure 5). There is no positive co-relation between frequencies of expression of these proteins and clinical characters except that tubular and cribriform type ACC which exhibited more frequently p53 protein overexpression ($p = 0.016$) (Table 7). The low expression of p53 phospho S15 was associated with worse ACC specific survival ($p = 0.05$), whereas overexpression of p53 was not significantly related to patient survival even it is related to decreased overall survival (Figure 6A and B).

Moreover, when analyzing ATM expression stratified by p53 and its ATM mediated phosphorylation status, overexpression of p53 with ATM loss significantly decreased overall survival ($p = 0.01$) and also low p53 phospho S15 expression with ATM loss has worse prognosis as shown in Figure 6C and D. These data supported that loss of ATM and its functional status in p53 pathway is an important factor associated with poor survival of patients.

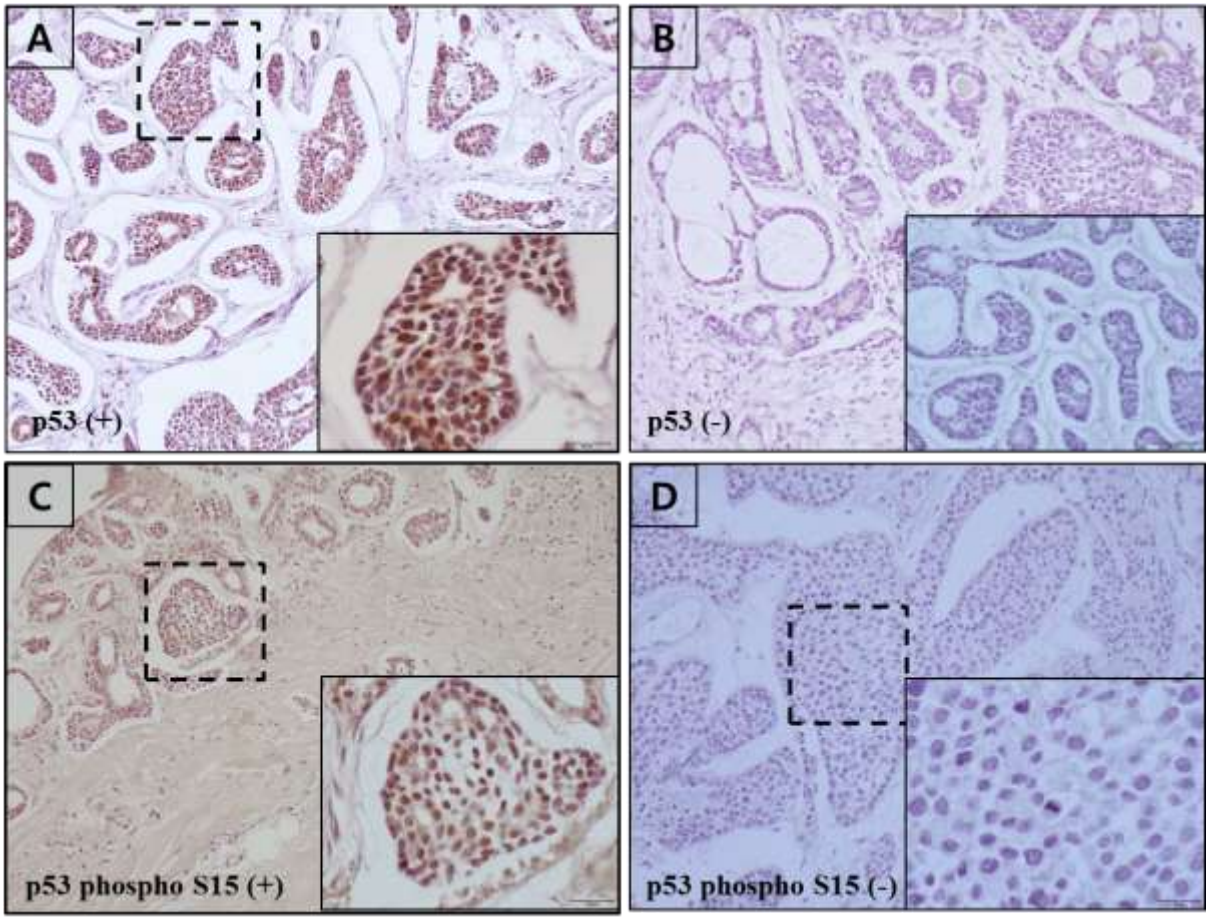

Figure 5. Immunohistochemical expression p53 and p53 phospho S15. (A) p53 positive expression (B) negative expression of p53. (C) p53 phospho S15 positive expression, (D) negative expression of p53 phospho S15.The original magnification of all figures was taken at ×200. Higher magnification (x1000) views.

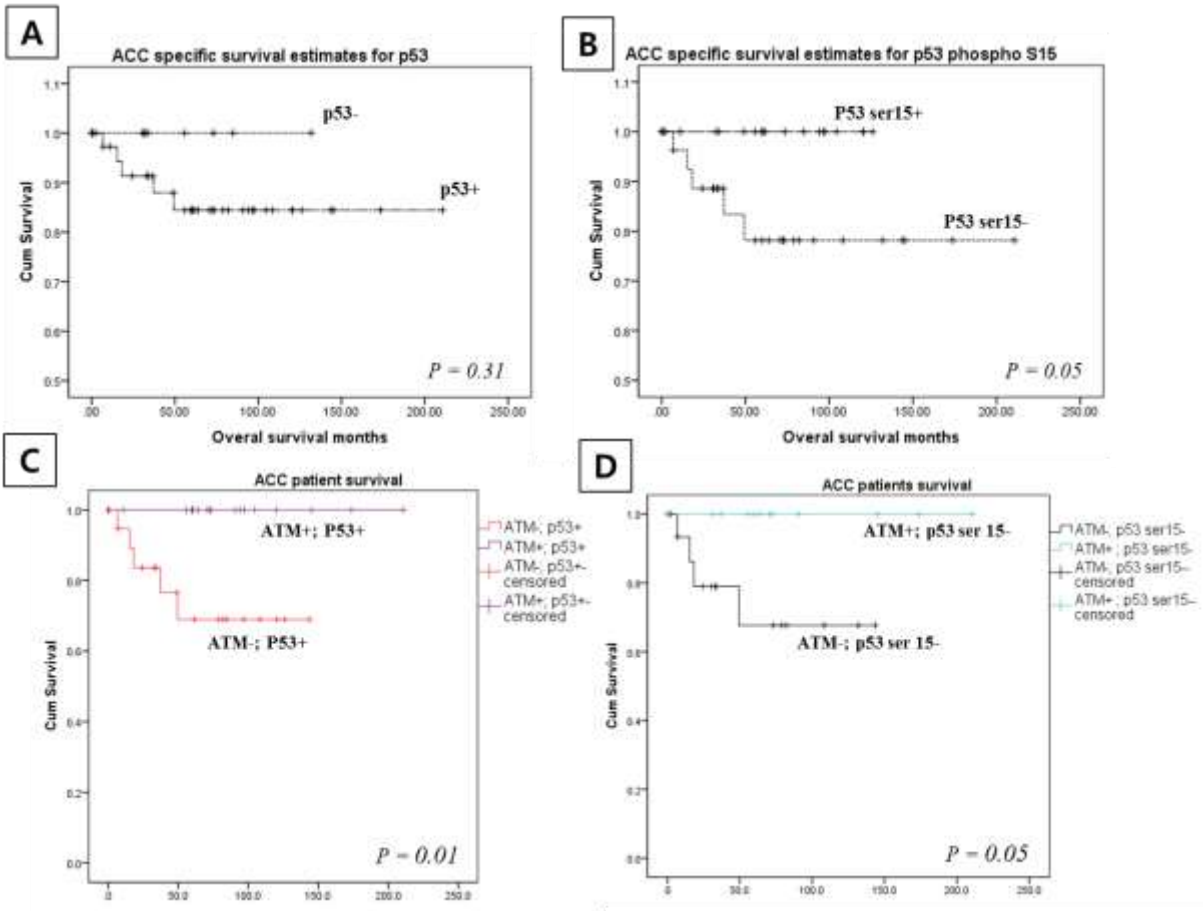

Figure 6. Overall survival of ACC patients according to p53, p53 phospho S15 protein expression and combination with ATM. (A) Overall survival according to p53 protein expression, (B) Overall survival according p53 phospho S15 expression, (C) Overall survival according combination of ATM and p53 protein expression (D) Overall survival according combination of ATM p53 phospho S15 protein expression.

5. Prognostic value of combination of ATM, p53, p53 phospho S15 expression and perineural invasion

We rather attempted to evaluate prognostic significance of combination of perineural invasion with expression of ATM and p53 and p53 phospho S15 individually and either of those coexpression status. As shown in Figure 7, none of them was found statistically significant.

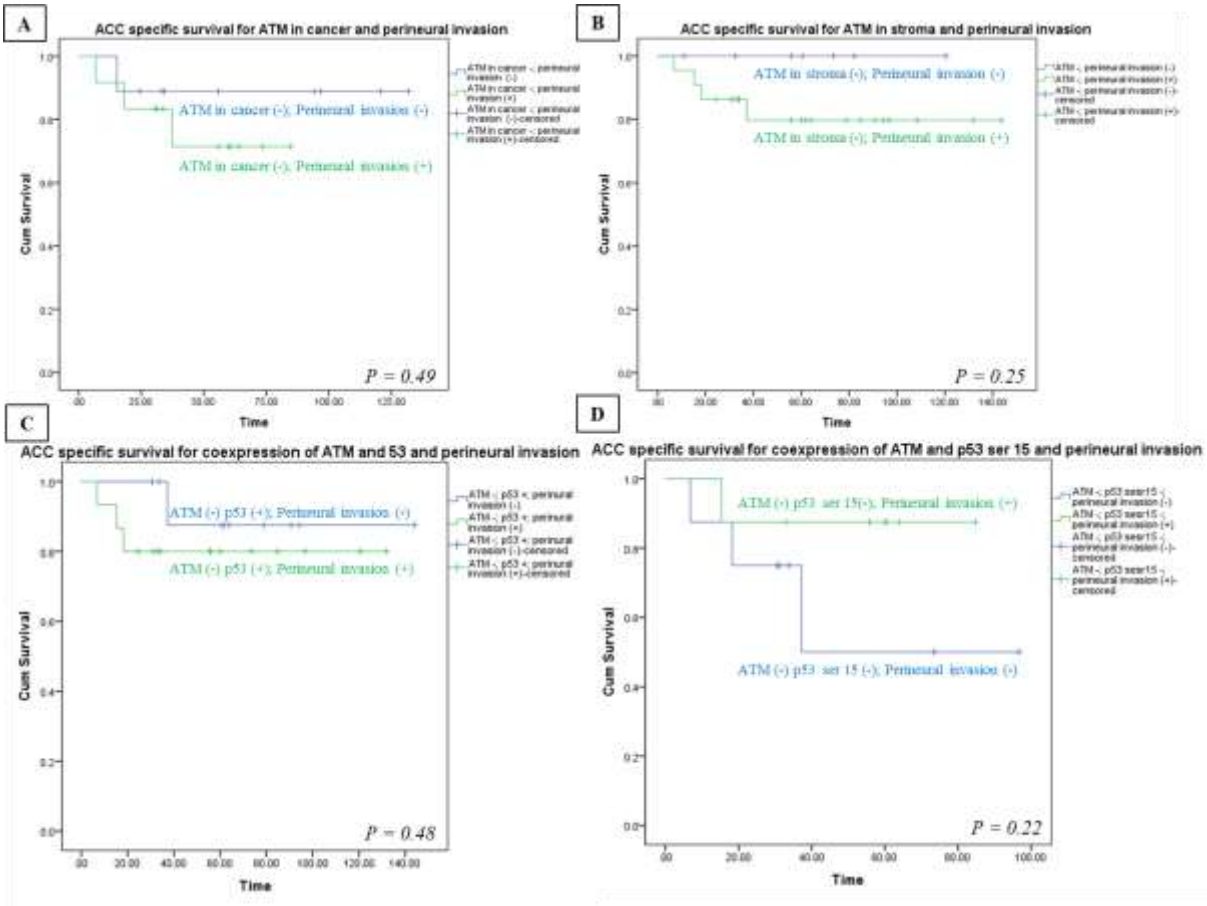

Figure 7. Overall survival according to ATM in cancer and perineural invasion in (A) and ATM in stroma and perineural invasion in (B) Overall survival according combination of ATM and p53 protein expression and perineural invasion in (C) Overall survival according combination of ATM p53 phospho S15 protein expression and perineural invasion in (D).

IV. DISCUSSION

The accumulation of genetic alteration contributes to tumor progression, metastatic phenotype, and grade of malignancy. LOH, which is indicative of the inactivation of tumor suppressor genes, has been observed at numerous chromosomal locations in many tumor types. There have been reported LOH at specific chromosome sites in several types of cancers such as colorectal cancers (5q, 17p, 18q) (Vogelstein et al., 1988), renal cancer and small-cell lung carcinoma (3p) (Chmara et al., 2004; Martinez et al., 2000), bladder cancer (9q, 11p,17p) (Tsai et al., 1990), and retinoblastoma (13q) (Zhang et al., 1994). In case of tumor progression of HNSCC, deletions on chromosome 3p, 9p, and 17p have been associated with the transition from normal mucosa to dysplasia, whereas carcinomas were characterized by additional deletions on 4q, 6p, 8, 11q, 13q, and 14q (Califano et al., 1996). In this study, highest frequency of LOH was found in chromosome 11q.

LOH in 11q23 is commonly reported in breast cancers (Carter et al., 1994) and in number of solitary tumors including colorectal carcinoma (Gustafson et al., 1994) cervical carcinoma (Bethwaite et al., 1995). One of gene located at 11q22.3 is the ATM gene and it has multiple complex functions including a central role in the repair of DNA double-strand breaks and signaling to cell cycle checkpoints, transcriptional regulation and activation of apoptosis (Du et al., 2015).

ATM is activated within seconds to minutes of exposure to ionizing radiation (IR) and can phosphorylate various downstream substrates, some of which are key factors in the regulation of cell cycle arrest, DNA repair, and apoptosis (Cremona and Behrens, 2014). For example, *ATM* is an upstream factor of tumor-suppressor protein TP53 and regulates progression of the cell cycle and apoptosis by activation and stabilization of *p53* (Canman et al., 1998). In response to ionizing radiation, the kinase activity of *ATM* is enhanced; leading to phosphorylation of *p53* on serine 15 also phosphorylates and activates the checkpoint kinase *Chk2*, which in turn, phosphorylates *p53* on serine 20. These interactions result in the stabilization of *p53* and its activation (Angele et al., 2000; Hirao et al., 2000). Moreover, serine 15, threonine 18 and serine 20 are key phosphorylation sites, and are involved not only in stimulating the interaction of *p53* with the transcriptional machinery, but can also inhibit the interaction of *p53* with MDM (Meek, 2009).

In this study, expression pattern of ATM, p53 and its ATM mediated phosphorylated phenotypes in ACC salivary glands and their expression pattern was an independent risk factor for patient's survival. Although abnormalities of individual double-strand breaks checkpoint repair proteins have been reported but most of them have been studied separately without considering the common pathway involved. The prognostic role of ATM and p53 expression have been demonstrated in other malignancies, including breast (Angele et al., 2000), pancreatic cancer (H. Kim et al., 2014) , lung (Villaruz et al., 2016), and gastric cancer (Kang et al., 2008). All these studies considered about independent ATM expression and its combinations with p53 expression were a risk factor for patient's outcome. In contrast, this present study evaluated (i) ATM serves as upstream sensor of double-strand breaks formation and phosphorylates p53 to trigger cell cycle regulation; (ii) joint contribution of ATM functional pathway may act together, leading to poor outcome of ACC patients.

There are several possible explanations for findings that overexpression of 53 was related to decreased worse survival even it was not statistically significant, whereas low expression of p53 phospho S15 was associated with worse survival ($p = 0.05$). First, mutations in p53 occurs almost half of human cancers and the mutational status of p53 is independent unfavorable prognostic marker in many malignancies (Miller et al., 2005). Mutated p53 that affect its conformation typically increase its half-life, in part by inhibiting degradation by the ubiquitin complex (Maki et al., 1996). Therefore overexpression of p53 can be coordinated by mutational status of this protein itself or it can be activated by ATM independent pathway. Second, the relationship between poor survival and low expression of p53 phospho S15 can be explained by Kastan et al study which revealed that in response to DNA damage, cells with wild-type ATM accumulate p53 protein and show a subsequent increase p53 activity, whereas cells with defective ATM show a smaller increase the amount of p53 protein in response to irradiation (Kastan et al., 1992). Therefore, ATM appears to act upstream of p53 in a signal, defective activation or loss of ATM cannot stabilize p53 at serine 15 which may leads poor survival of patients. Meanwhile, the loss or reduced expression of ATM and may lead to the irreparable damage to DNA, thus easily causing the cancerization of cells, and losing control of its own differentiation and proliferation at the gene level, ultimately inducing diseases progression (Han et al., 2017). ATM, Chk2, and p53 activation are characteristic of early stages of tumorigenesis, with

subsequent checkpoint silencing and escape during tumor progression (Bartkova et al., 2005). Jiang et al revealed that inactivation of any one of ATM and p53 proteins is sufficient to disable this early tumor checkpoint (Jiang et al., 2009). Therefore these results suggested that combined alteration of those two protein result in poor survival.

Several authors have retrospectively studied the clinico-pathological features of ACC, attempting to identify significant prognostic factors, but the findings still remain controversial. Solid histological and perineural invasion have been considered as important indicators of poor outcome of ACC patients. The presence of perineural invasion can result in a high rate of recurrence (Chen et al., 2006; Min et al., 2012). Of note, in these study loss of ATM in stromal fibroblasts was found significantly related to perineural invasion (p=0.008). This data suggests that loss of ATM in stromal fibroblasts is important signaling high rate recurrence. Feng et al study demonstrated that reduced ATM expressions in malignant tumor as well as cancer associated stroma are independent prognostic marker in breast cancer {Feng, 2015 #139. They observed that low ATM within malignant breast epithelial and stroma is associated with aggressive characteristics of breast cancer. Therefore we believe that these data suggets low expression of ATM in stromal fibroblast may be associated tumor invasion and distant metastasis. However, there is no correlation between perineural invasion and poor prognosis in this study. This study attempted to eveluate prognostic significance of combination of perineural invasion with expression of ATM and p53 and p53 phospho S15 individually and either of those coexpression status. None of them was found statistically significant. In Figure 7D, combination of loss of ATM, p53 phospho S15 and perineural invasion shows better prognosis compared to patients who have loss of ATM, p53 phospho S15 and nonperineural invasion. It might be caused by small number of study population. Further validation will be required using a larger distinct study cohort.

Supported by previous studies, Myb-NFIB fusion was not expressed in non-ACC neoplasms of the head and neck, confirming the high specificity of the Myb-NFIB fusion for adenoid cystic carcinoma (Brill et al., 2011). A series of all of 48 ACC patients sample were screened for Myb staining. 82% of whole population of our study groups was stained positively for Myb, whereas not detected in non-tumoral salivary gland parenchyma. Myb expression difference according to perineural invasion and

worse outcome showed no significance. Therefore, Myb may be helpful marker to distinguish ACC from other salivary glands but not prognostic marker.

Limitations of our current study might be considered as follows. First, small number of study population in both cohorts which included only 5 death cases. Second, the detection method and interpretation for p53 phospho S15 expression should also be defined through validation study to apply p53 phospho S15 expression to other population.

Considering the interconnecting network of multiple regulatory genes and genetic instability in cancer development, further prospective studies should be warranted by combined study of genetic stability and protein expression with the same gene in large number study groups.

V. Conclusion

Our study supports that ATM and ATM dependent p53 pathway may act together and may lead to poor survival of ACC of salivary glands.

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Abstract (Korean)

선양 낭성 암종에서 ATM 단백질 발현을 통한 예측 인자 분석

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1. 서론

두경부 선양낭성암종은 높은 재발과 긴 기간에 걸친 원격 전이를 특징으로 하는 타액선의 악성 종양 중 하나이다. 최근, Myb-NFIB 유전자 융합 또는 Myb의 재조합이 선양낭성암종의 특징으로 검출되었다. 그러나 선양낭성암종의 예후를 예측하는 생물학적 표지자는 아직 발견되지 않았다. 세린 / 트레오닌 단백질인 ataxia telangiectasia mutated (ATM) 은 DNA 이중 가닥 절단을 복구하는 복잡한 과정에서 핵심적인 역할을 하여 유전체 항상성을 유지한다. 이전까지, 타액선 종양에서의 ATM 발현은 보고 된 바가 없다. 본 연구에서는 선양낭성암종의 암 조직과 암 주위 기질조직에서의 ATM 유전자의 불안정성 및 단백질 발현 상태를 분석하고 예후와 생존율과의 관련성을 밝혔다.

2. 연구대상 및 방법

실험 목적에 따라 두 집단의 코호트를 분석하였다. 첫 번째 집단은 11 명의 선양낭성암종 환자의 조직으로 DNA 추출과 이형접합성 상실 (loss of heterozygosity) 분석을 시행하였다. 염색체 9p, 11q, 21q에 대하여 이형접합성 상실 분석을 하였다. 두 번째 집단은 48명의 선양낭성암종 환자의 조직으로 ATM 발현 및 ATM의 하향조절인자인 p53 발현의 분석을 시행하였다. 이 연구를 위하여 면역조직화학염색을 시행하였으며, 카플란 마이어 분석으로 단백질 발현률과 환자의 생존율과의 연관성을 평가하였다.

3. 결과

첫번째 이형접합성 상실 연구결과는 D11S1778 영역에서 암세포와 암 주위 기질 섬유모세포의 LOH 빈도가 가장 높았다. D11S1778의 LOH는 11예 중 암 세포에서 4 예, 암 주위 기질 섬유모세포에서 5 예에서 발견되었다. 현미부수체 불안정성 (MSI) 와 이형접합성 상실 은 환자 2명의 암세포와 암 주위 기질 섬유모세포에서 동시에

발견되었다. D11S1778 영역에 있는 유전자 중 암발생 관련성이 높은 유전자인 Ataxia telangiectasia mutated (ATM) 유전자를 선택하였다. 따라서, 이 연구는 선양낭성암종에서의 ATM과 ATM의 하향 조절 단백질 발현의 예후 효과를 조사하는데 초점을 두었다. 결과적으로, 암세포에서의 ATM의 낮은 발현을 가진 환자는 정상 조직과 동일한 ATM 발현을 갖는 환자와 비교하였을 때 낮은 생존율을 보였다. 그러나 암 주위 기질 섬유모세포에서의 ATM의 발현은 환자의 생존율과 유의한 연관성이 없었다. 또한, ATM의 낮은 발현을 가진 환자를 p53 발현 상태 또는 ATM 매개 p53 인산화 Ser15 발현 상태에 따라서 나누어 분석을 시행하였다. 그 결과, ATM의 낮은 발현을 보이는 환자들 중에 비정상적인 p53의 과발현을 보이거나 (p=0.01) p53 인산화 Ser15 낮은 발현을 갖는 환자에게서 생존율이 유의하게 감소되었다 (p=0.05). 기존에 예후 예측인자로 알려진 신경조직 침윤소견은 예후와의 연관성을 볼 수 없었으며, ATM 발현소실, p53 과발현과 함께 분석하여도 생존율 예측에 도움이 되지 않았다.

4. 결론

이 연구결과로 보아 두경부 선양안성암종에서 ATM 발현 소실과 p53 과발현은 예후를 예측하는 분자생물학적 표지자로 가능하다고 판단되었다.

중심단어: 선양낭성암종, ATM, 이형접합성 상실