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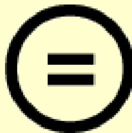

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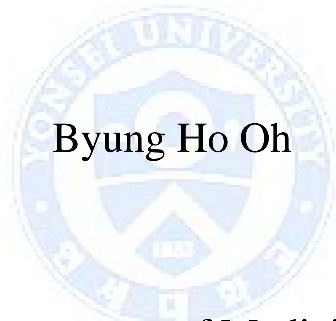
Developing an aging index to predict the occurrence of non-melanoma skin cancer in Koreans



Byung Ho Oh

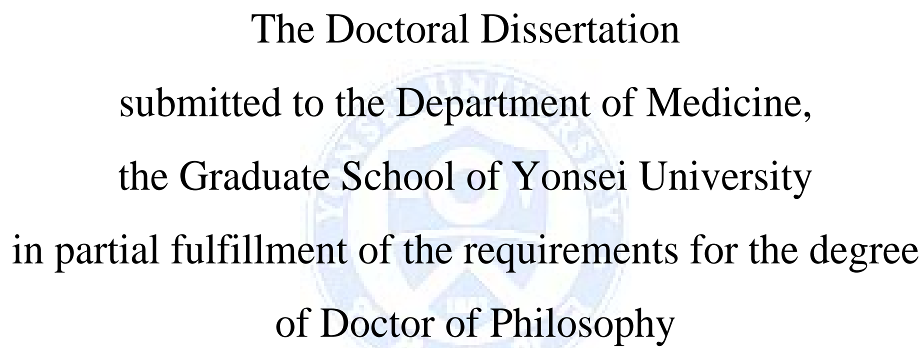
Department of Medicine

The Graduate School, Yonsei University

Developing an aging index to predict the occurrence of non-melanoma skin cancer in Koreans

Directed by Professor Kee Yang Chung

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy



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June 2015

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ABSTRACT

Developing an aging index to predict the occurrence of non-melanoma skin cancer in Koreans

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(Directed by Professor Kee Yang Chung)

Although new anti-aging cosmeceuticals and cosmetic procedures are continuously developed, a skin aging index for an objective assessment has not been clearly proposed. Among the many mechanisms involved in the aging process, mitochondria with reactive oxygen species are closely linked to human aging. A single cell has approximately 100 to 10,000 copies of mitochondrial DNA which tend to accumulate as heteroplasmy due to a limited capacity for DNA repair. Thus, the degree of aging might be calculated from the amount of the mitochondrial DNA damage.

The aim of this study is to suggest a skin aging index based on the quantitative analysis of deletions in certain regions of mitochondrial DNA, and to investigate whether the index can predict the occurrence of non-melanoma skin cancer (NMSC). Mito-resequencing assay showed that there were 28 and 11 "N" points, which are in positions where not more than 50%

of sequences were detected, in squamous cell carcinoma (SCC) samples and basal cell carcinoma samples, respectively. In SCC samples, the “N” points were detected in proximity to 888, 896, 899, and 902-base pair (bp). Next, I designed a new primer for the quantitative measurement of deletions at these points. As a result, the amount of new deletions was significantly higher in the skin of SCC than peri-lesional normal skin (21.77±0.46 vs. 20.62±0.40, p<0.001). In addition, it was significantly higher in the peri-lesional normal skin of 76 NMSCs than that of 68 benign tumors (22.68±1.81 vs. 21.71±1.95, p=0.002). However, there was no significant correlation between age and the new deletions in these subjects. Although the amount of 4977-bp deletion was significantly related with age, its application in clinical setting might be limited due to large standard deviation (Pearson correlation coefficient=-0.195, p=0.019). In multivariate analysis, the amount of new deletions and occurrence of skin cancer were related, as the odd ratio for the decrease in the amount of deletion and the risk of NMSC was 0.709. In conclusion, mitochondrial DNA deletion on the 888, 896, and 902-bp can predict the occurrence of non-melanoma skin cancer in Koreans, and further analysis of these new deletions is required for a development of a skin aging index.

Key words: mitochondrial DNA, skin aging index, non-melanoma skin cancer, common deletion, mito-resequencing assay

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I. INTRODUCTION

Skin aging is a complex process that consists of chronologic(intrinsic) aging associated with people's genetics and extrinsic aging associated with ultraviolet rays, alcohol consumption, smoking, poor nutrition, and adverse environmental conditions.[1] Although new developments on cosmeceuticals and cosmetic procedures have been made with an increasing interest in anti-aging, a skin aging index which makes objective assessment possible has not yet been clearly proposed. In current clinical practice, although the photographic staging scales and clinical assessments such as SCINEXA (Score for Intrinsic and Extrinsic skin Aging) are used to measure skin aging quantitatively, all these scales remain insufficient in establishing all effects of

skin aging, regional assessment, and effectiveness of treatments.[2-7] For more objective assessment, the use of dermoscopy or the measurement of melanin and facial skin fluorescence has been suggested,[8,9] but objective quantification is still not plausible.

The ideal scale to evaluate the aging of skin should be simple, easily applicable, non-invasive, capable of quantitative evaluation, and easily used both in clinical and epidemiological studies.[8] Furthermore, it must enable the prediction of occurrence of skin cancer, which is the most serious complication of skin aging. For this, it has to reflect DNA damage caused by tissue oxidation ultimately, which is the most fundamental issue underlying skin aging.

With the evolution of molecular biology, it was recently proposed that the accumulation of mitochondrial DNA (mtDNA) damage has been strongly associated as an underlying cause of the general aging process in tissues and mtDNA damage has been associated with cancer development in the ovary, breast, etc.[10-12] In the case of skin, most investigations have been the analysis of 3895 and 4977-base pair (bp) mtDNA deletion which has been found to be related to photoaging.[13-15] However, it is still unclear whether the mutations of mtDNA have a direct bearing on carcinogenesis in skin, and there also have been scarce reports in Asian subjects with Fitzpatrick skin type IV-V.

Under the hypothesis that mtDNA damage has a relation with aging and skin cancer development, the differences of mtDNA damage between non-

melanoma skin cancer (NMSC), such as squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), and their peri-lesional normal skin were analyzed to find more specific sites of mtDNA deletion in NMSC. In addition, a quantitative analysis of various mtDNA deletions depending on the body sites and age groups was performed to propose a highly reliable skin aging index which can predict the occurrence of NMSC in Korean populations.

II. MATERIALS AND METHODS

1. Method for finding the changes of mitochondrial morphology with aging

To find the differences of mitochondrial morphology with aging, electron microscopy of skin samples from the buttocks of 15 and 78 year old male patient was conducted. Each sample was fixed with 2% Glutaraldehyde - Paraformaldehyde in 0.1M phosphate buffer(PB), pH 7.4 for two hours and washed three times for 30min in 0.1M PB. They were fixed with 1% OsO4 dissolved in 0.1M PB for two hours and dehydrated in ascending gradual series (50 ~ 100%) of ethanol and infiltrated with propylene oxide. Specimens were embedded by Poly/Bed 812 kit (Polysciences). After pure fresh resin embedding and polymerization at 60℃in an electron microscope oven (TD-700, DOSAKA, Japan) for 24 hours 350 ㎚ thick sections were initially cut and stained with toluidine blue for observation with a light microscope. 70 ㎚ thin sections were double stained with 7% (20min) uranyl acetate and lead citrate for contrast staining. There sections were cut by LEICA EM UC-7 Ultra-microtome (Leica Microsystems, Austria). All of the thin sections were observed by transmission electron microscopy (JEM-1011, 80Kv JEOL, Japan) at the acceleration voltage of 80 ㎸.

2. Method for finding a specific mtDNA deletion of non-melanoma skin cancer patients

A. Skin samples for array-based analysis of mtDNA alterations

Four non-melanoma skin cancer cases of the facial region with matched peri-lesional normal samples (two from SCC patients, two from BCC patients) and four normal skin samples from BCC patients, totaling 12 specimens overall, were accrued as follows (Table 1).

Table 1. Characteristics of skin samples.

Case no.	Sample no.	Age	Sex	Diagnosis	Samples
1	1	40	F	BCC	PNS
2	2	58	F	BCC	PNS
3	3	61	M	BCC	PNS
4	4	65	M	BCC	BCC
	5			BCC	PNS
5	6	67	F	BCC	PNS
6	7	74	F	BCC	PNS
	8			BCC	BCC
7	9	80	M	SCC	SCC
	10			SCC	PNS
8	11	88	M	SCC	SCC
	12			SCC	PNS

F: Female, M: Male, SCC: Squamous cell carcinoma, BCC: Basal cell carcinoma, PNS: Peri-lesional normal skin.

B. DNA extraction and quantification

DNA was extracted from the skin samples using a QIAamp DNA Mini kit (Qiagen, Crawley, U.K.), automated on a QIAcube (Qiagen) using standard buccal swab protocols. The extracted DNA was then quantified with a Qubitfluorometer (Invitrogen, Paisley, U.K.) using a Quant iT Pico green dye kit (Invitrogen).

C. Mito-resequencing array

To find a specific mtDNA deletion of non-melanoma skin cancer (NMSC) patients, mito-resequencing array by Affymetrix's MitoChip v.2.0 was performed. The MitoChip is based on microarray technology that contains 25-mer probes complementary to the revised Cambridge Reference Sequence (rCRS).[16] In addition, it revealed an average call rate of 99.48% and an accuracy of > 99.98%.[17] All samples were prepared for resequencing according to the Affymetrix GeneChip® Resequencing Assay kit (P/N900447) manual and GeneChip® CustomSeq® Resequencing Array Protocol v.2.

DNA amplification was performed independently using three primer pairs previously used for fluorescent DNA sequencing resulting in 3 amplicons of 3–7kb length for full coverage of the mitochondrial genome. Each PCR product was visualized on an agarose gel and accessed amount of PCR products by the ND-1000. The mitochondrial DNA template, 10 μmol of

primers, 0.5U LA Taq polymerase (TaKara), 5 μL buffer, 8 μL dNTPs (10 μmol each) and 33μL of dH20 were mixed for a total reaction volume of 50μL. Thermal cycling conditions were as follows: 94°C for two minutes, followed by 30 cycles of 94°C for 15 s, 68°C for eight minutes; final elongation 68°C for 12 minutes; 4°C hold. The three PCR products for each subject were purified in plate format. Then purified PCR amplification products were analyzed for quality and quantity as done previously or by spectrophotometric methods. Subsequently, each DNA was fragmented to less than 20~200-bp using partial DNase I digestion. After the fragmentation step the products were end-labeled with a biotinylated nucleoitde using Terminal Deoxynucleotidyl Transferase. The fragmented end-labeled products were added to a hybridization solution, denatured, and added to a GeneChip® Mitochondrial resequencing Array 2.0 (one array per sample) for 16 hours at 45°C and 60 rpm as described in GeneChip® CustomSeq® Resequencing Array Protocol v.2. After hybridization, the chips were stained and washed in a Genechip® Fluidics Station 450 and scanned by using a Genechip® Array scanner 3000 7G.

D. Data Analysis

Cell intensity (CEL) files and raw sequence data were generated using GeneChip® Command Console® Software 4.1.2 (AGCC 4.1.2; Affymetrix) and GeneChip Sequence Analysis Software 4.0 (GSEQ 4.0; Affymetrix),

respectively. Since algorithm parameters affect both the call rate and the accuracy of the analysis, the call rate of the analysis result was appropriately maximized by adjusting the empirical Quality Score threshold and the base reliability threshold (BRT) options. Data were analyzed using the haploid model with a quality score thresholds 12 (QSTs 12). The QST filters out calls with a score lower than the respective threshold and reports them as ‘‘no-calls.’’

In addition, the analysis was performed with or without the base reliability threshold (BRT) set at 0.5; a position must be detected as> 50% of the sequences, otherwise it is designated as “N” in all samples. To ensure reliable detection of variants occurring in close proximity to each other (≤ 12 bases), the MitoChip v2.0 is tiled with additional probes that are complementary to common variants in the HVR1, HVR2, and the coding regions (nps 48–361, 726–774, 1414–1462, 4745–4793, 8836–8884,15302–15350, and 16045–16386).

3. Gene-specific primer design to detect specific mtDNA deletion of NMSC patients

Based on the results of mito-resequencing array, gene-specific primer was designed using Primer Blast (National Center for Biotechnology Information, Bethesda, MD, U.S.A.). Because detection of a position was made possible

only when there was more than 50% of the sequences on mito-resequencing array, it was predicted that positions marked "N" contained a high copy number of mtDNA deletions. Therefore, gene-specific primer was designed from a segment where "N" points were detected frequently in proximity.

4. Method for quantitative analysis of various mtDNA deletions depending on the body sites, age groups, and the presence of NMSCs

A. Skin samples for mtDNA analysis

144 peri-lesional normal skin samples were corrected from 76 patients of NMSCs and 68 patients of benign tumors such as lipoma, epidermal cyst, etc. to find a difference of mtDNA deletion amounts among various body sites, and ages. To compare the amount of mtDNA deletion of SCC from peri-lesional normal skin, lesions and peri-lesional normal skin samples were corrected from the 21 patients of SCC. This study protocol was approved by the Institutional Review Board of Yonsei University (IRB No. 4-2014-0200), Severance Hospital and was conducted according to the Declaration of Helsinki Principles. In 73 samples collected from the facial area, SCINEXA score, which is a gross measurement of skin aging, was also obtained.[6]

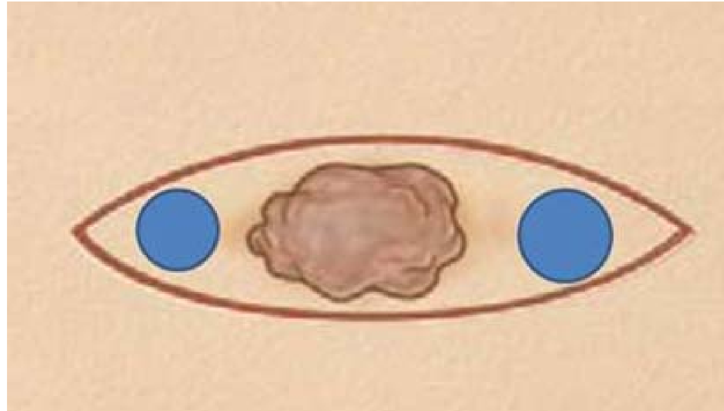


Figure 1. An example of normal tissue during the excision of benign or malignant skin tumor. The study was conducted with normal skin tissues around NMSC or benign tumors (blue circles), under informed and expressed consent of patients.

B. DNA extraction and quantification

DNA was extracted from skin tissues using a QIAamp DNA Mini kit (Qiagen, Crawley, U.K.). The extracted DNA was then quantified by the ABI 7500 system (Applied Biosystems), Real-time PCR amplifications were performed as total volume of 25uL reaction mixture containing 100ng genomic DNA template in a 96-well microplate (Applied Biosystems) and using SYBR Green ER Supermix with Premixed ROX (Invitrogen Life Technologies) in an ABI 7500 following the manufacturer's protocol. Gene-specific primers were designed using Primer Blast (National Center for Biotechnology Information, Bethesda, MD, U.S.A.). The common deletion sites were amplified using following primers: H8388F, F:5′-

ATGGCCCACCATAATTACCC-3´; H13477R, R:5´-GCTAATGCTAGGCTG-CCAAT-3´. Amplification conditions were as follows: initiation at 50 °C for 2 min, 95 °C for 10 min, followed by cycling conditions of 95 °C for 10 s and 60 °C for 1 min for 40 cycles. Following amplification, melting curve analysis was performed from 50 to 105 °C, reading every 0.5 °C. For quantitative analysis cycle threshold number (CTN) of real-time PCR was assessed. Under the current scenario, the CTN value becomes smaller as the number of damaged mtDNA copies at segments where deletions have occurred increases. For example, a CTN difference of 3 is equivalent to a theoretical change of 2^3 in the amount of DNA template.

C. Statistical analysis

Independent sample t-tests were carried out to compare the differences between two groups. When comparing lesional samples with samples of normal surrounding skin samples, the Wilcoxon Signed Ranks Test, a non-parametric test, was used. Pearson's correlation was used to examine the correlations between the amount of deletion and ages. In multivariate analysis, logistic regression analysis was performed to examine the odds ratio (OR) for having a NMSC. For all analyses, $P < 0.05$ was determined to be statistically significant.

III. RESULTS

1. Electron microscopy of mitochondrial morphology with aging

In comparison of skin on the buttocks, which is a non-exposed site, basal layer keratinocytes of a 15 year-old man exhibited normal morphology of the outer membrane, the intermembrane space, the inner membrane, and the cristae and matrix, whereas that of a 78 year-old man showed two variants of change. One is typical cristae disappearance, and the other is inner membrane vesiculation (Figure 2). From this result, it could be inferred that mitochondrial changes are closely related to skin aging.

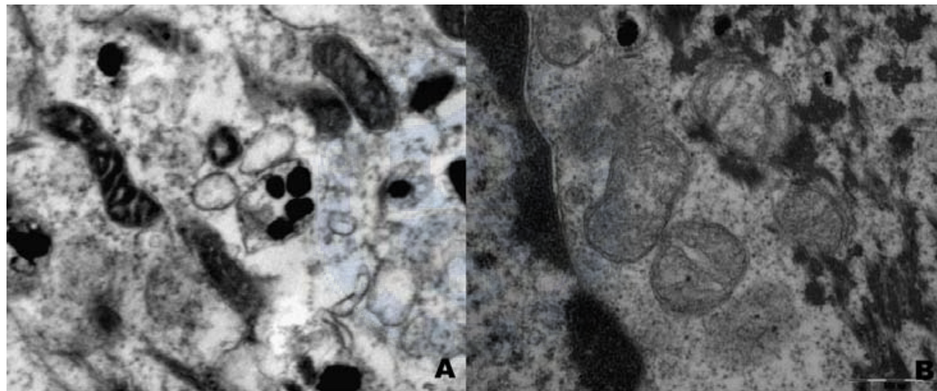


Figure 2. Electron microscopy of mitochondria. (A) An image showing several normal mitochondria from a fifteen year old male patient (x20k) (B) A sample from a 78 year old male patient showing typical cristae disappearance and inner membrane vesiculation (x20k).

2. Array-based analysis for mtDNA alterations

From the mito-resequencing array, the base sequences from 13 to 16557 were identified. Call rate, not taking into account the ones designated “N” (not detected because there was less than 50% of the sequences) varied between 95.72 to 96.42%, depending on samples (Table 2). When the outcome of sequencing with Revised Cambridge Reference Sequence (rCRS) was compared, there were 18 to 37 base changes of mtDNA depending on samples. Hypervariable segment was the region where most mtDNA alterations were observed (Figure 4). However, there was hardly, if any, differences between NMSC and surrounding normal tissue in the value of mtDNA alteration (Table 2).

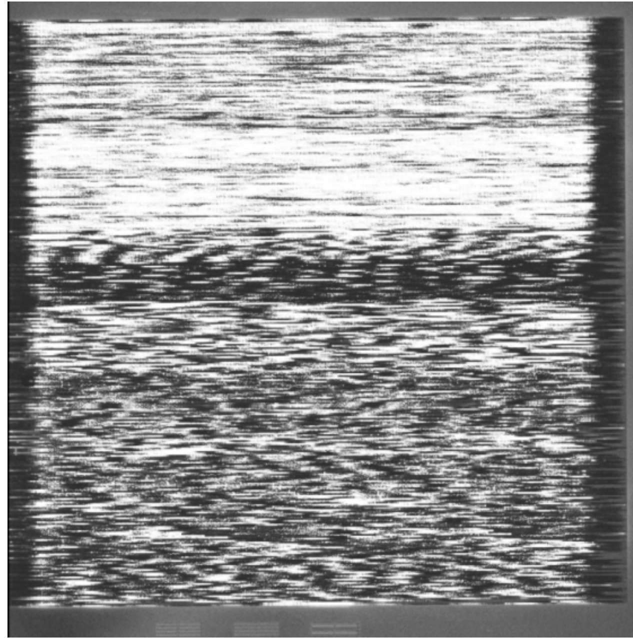
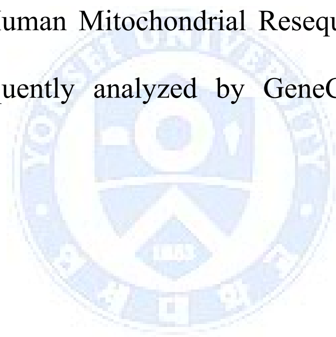


Figure 3. GeneChips Human Mitochondrial Resequencing Array of sample no.1. This was subsequently analyzed by GeneChip Sequence Analysis Software.



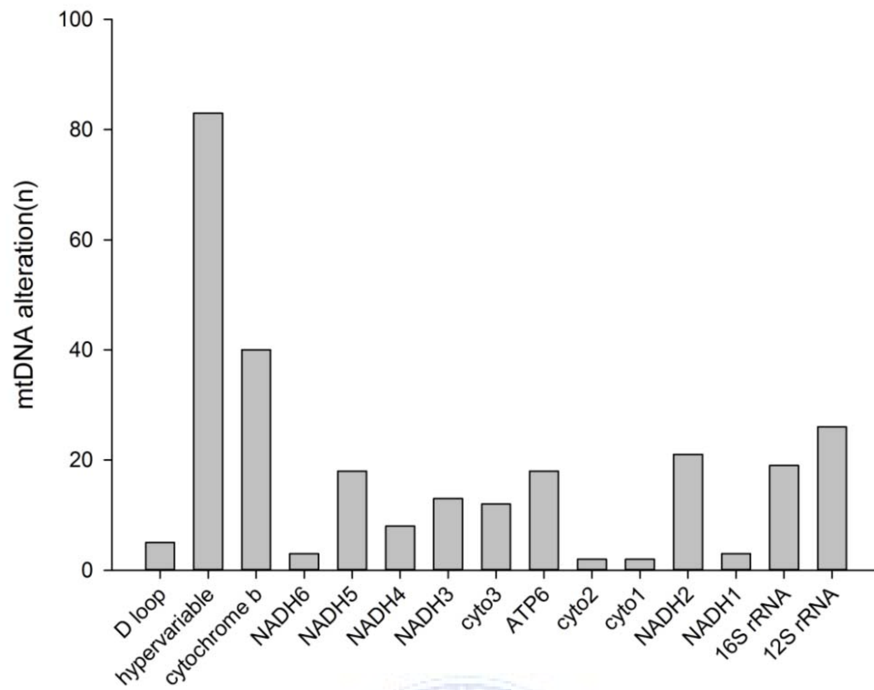


Figure 4. Distribution of mtDNA alterations. On comparing the outcome of sequencing with Revised Cambridge Reference Sequence (rCRS), there were 18 to 37 base changes of mtDNA depending on samples. Hypervariable segment was the region where most mtDNA alterations were observed.

Table 2. Results of mtDNA alteration and call rate. There was no difference in mtDNA alteration among samples 4-5, 7-8, 9-10, 11-12, all from the same subject.

Sample no.	Age	Sex	Diagnosis	Samples	mtDNA alteration (n)	Call rate (%)
1	40	F	BCC	PNS	32	96.07736476
2	58	F	BCC	PNS	37	96.48836506
3	61	M	BCC	PNS	20	95.72076156
4	65	M	BCC	BCC	18	96.40374736
5			BCC	PNS	19	96.16198247
6	67	F	BCC	PNS	18	95.74493805
7	74	F	BCC	PNS	23	96.21637957
8			BCC	BCC	23	95.92021759
9	80	M	SCC	SCC	26	96.18011484
10			SCC	PNS	27	96.20429133
11	88	M	SCC	SCC	23	95.92626171
12			SCC	PNS	22	96.42792384

F: Female, M: Male, SCC: Squamous cell carcinoma, BCC: Basal cell carcinoma, PNS: Peri-lesional normal skin.

3. Comparison of “N” points on array-based analysis

The points designated “N” (not detected because there was less than 50% of the sequences) between NMSC and normal skin tissues were investigated. Consequently, a total of 28mtDNA points were observed with “N” in SCC lesions compared to peri-lesional normal skin (PNS): 13984, 11774, 11234, 10177, 9775, 9571, 9390, 8083, 8069, 7863, 7855, 7388, 7233, 6880, 6266, 6267, 6037, 3927, 3661, 1592, 1462, 522, 517, 309. In particular, four “N” points such as 888, 896, 899, 902 in the 12S ribosomal RNA portion were found in the SCC samples (Figure 5). Because an “N” was found in proximity, it was predicted that there would be the greatest difference in mtDNA copy numbers between SCC and normal skin tissues in this site. In BCC lesions, a total of 11 mtDNA points were observed with “N” compared to PNS: 15535, 14807, 14487, 12486, 11867, 10950, 8431, 6261, 4970, 3897, 961. In BCC samples, there were no segments where “N” points were found nearby.

```
Seq Pos
Reference  ttcgtgccagccaccgcggtcacacgattaaccca
SCC >>     ttcgnnncngccancnnggncacacgattnaccca
PNS >>     ttcgngncagccaccgcggtcacacgattaaccca
PNS >>     ttcgngncagccaccgcggtcacacgattaaccca
SCC >>     ttcgnnncagccancgnggncacacgattaaccca
```

Figure 5. Adjacent "N" points such as 888, 896, 899, 902 in the 12S ribosomal RNA portion compared to the peri-lesional normal skin. It was deemed necessary to check the number of deleted mtDNA copies because a "N" was found nearby these points. SCC: Squamous cell carcinoma, PNS: Peri-lesional normal skin.

4. Quantification of deletion amount in SCC skin samples compared to the peri-lesional normal skin

Based on the results of mitochondrial resequencing array, a new primer was designed which can detect new deletions (888, 896, 902-bp) of mtDNA (F:5´-CGGCCAGCCCCGCGTCACA-3´, R:5´-TGGAGTTTTTACAACTC-AGGTG-3´). From the results of mtDNA deletion quantification using RT-PCR from the skin samples of 21 SCC patients, much more deleted amounts were detected in the SCC lesions than peri-lesional normal skins (Figure 6). In the statistical analysis, significant decrease of CTN value was found in SCC

lesion compared to peri-lesional normal skin (21.77±0.46 vs. 20.62±0.40, $p<0.001$). However, there was no significant difference in the amount of 4977-bp deletion between SCC lesion and normal skin tissues ($p=0.741$)(Figure 7).

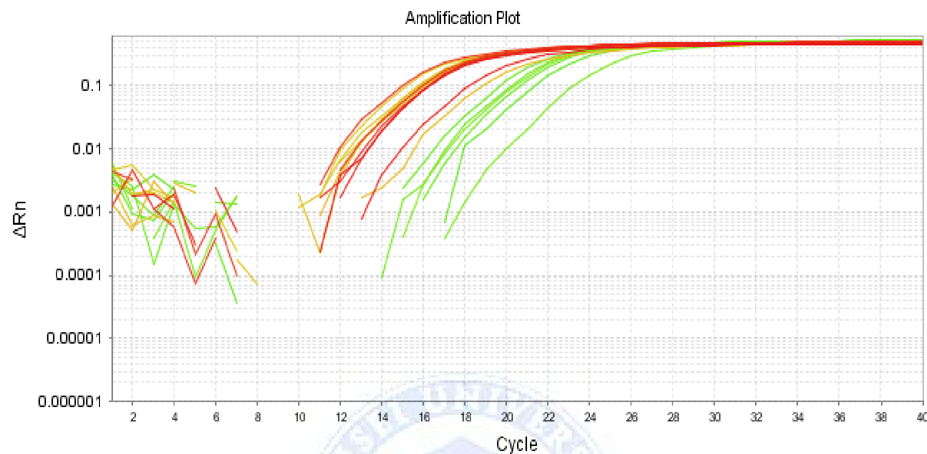
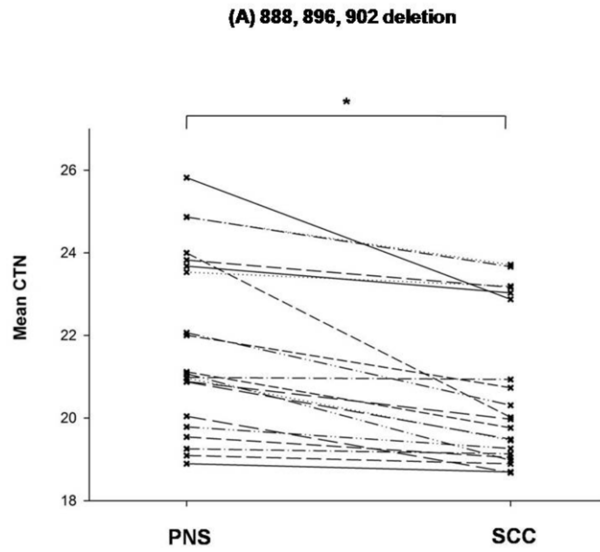


Figure 6. mtDNA deletion quantification using RT-PCR. The cycle threshold number was much higher in the peri-lesional normal skin (green color) than SCC samples (red color), which means that SCC lesions have much copy numbers of new mtDNA deletion (del888, del896, del902).



* p<0.001, Wilcoxon Signed Ranks Test

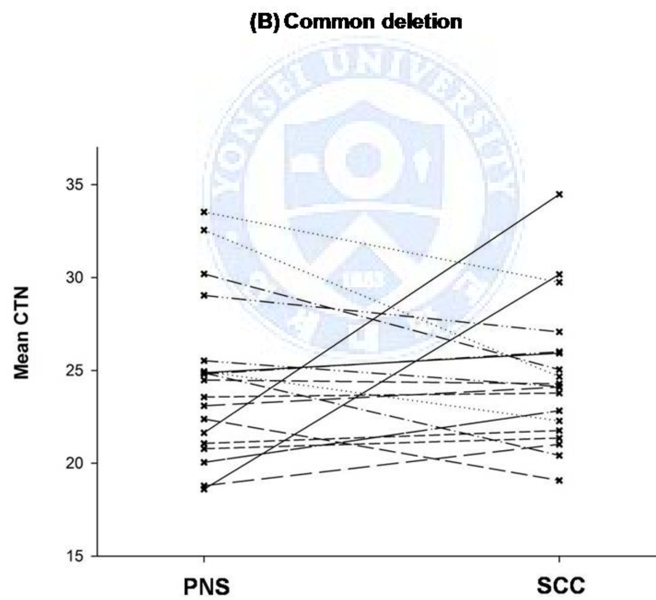


Figure 7. (A) Comparision of mtDNA deletion (del888, del896, del902) quantification between peri-lesional normal skin (PNS) and squamous cell

carcinoma (SCC). **(B)** Comparision of common deletion quantification between PNS and SCC. The amount of deletions at 888, 896, 902-bp using newly designed primer showed significant difference statistically, but that of common deletion did not (Wilcoxon Signed Ranks Test, $p<0.001$ vs. $p=0.741$).

5. Difference in the amount of deletion according to the age of patients

The next step of this study is to determine any difference in mtDNA copy numbers of deletions according to age by an analysis of 144 peri-lesional normal skin samples. The age of the 144 patients ranged from 6 to 93, and the mean age was 55.30 ± 22.39. No significant difference in del888, del896, del902 quantification in terms of age was found (Figure 8A). In contrast, with respect to common deletion of 4977-bp, the age of subjects showed inverse correlation with mean CTN, and thus increase in the amount of deletion (Figure 8B) (Pearson correlation coefficient = - 0.195, $p=0.019$).

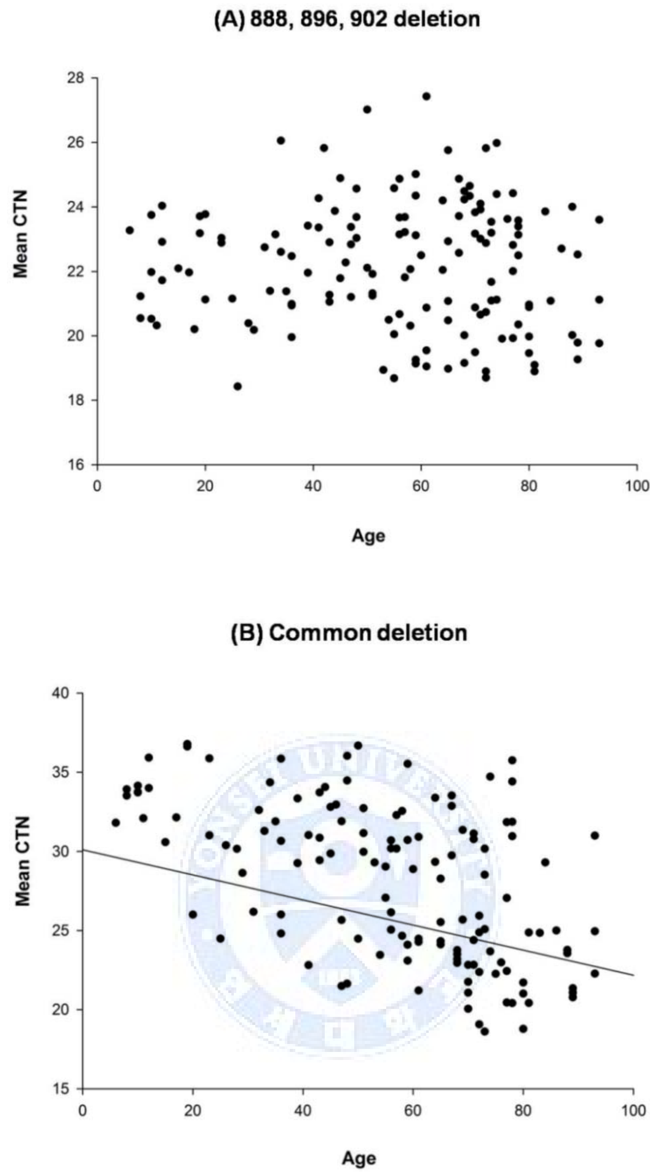
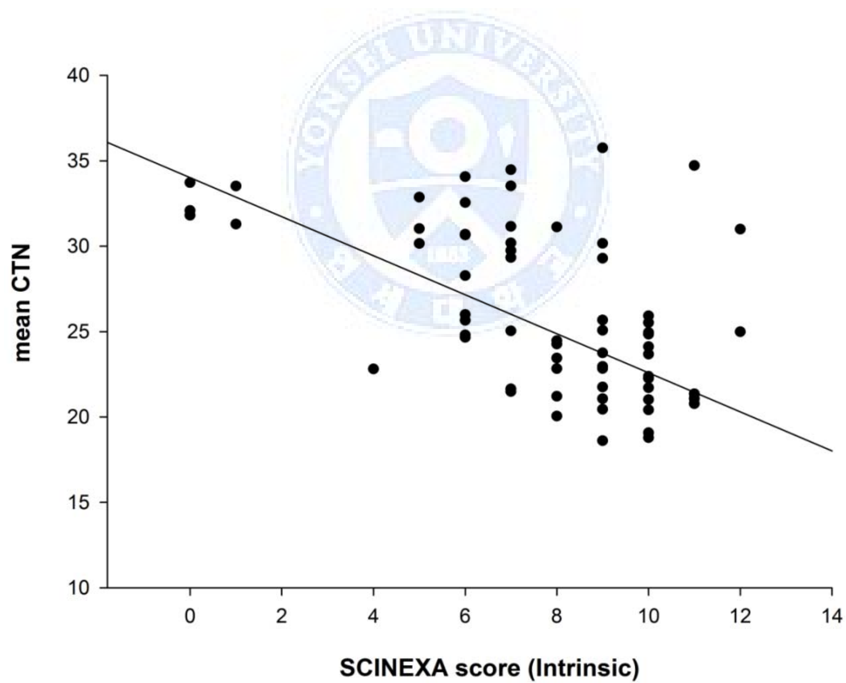


Figure 8. Difference in the amount of mtDNA deletion in terms of the age of patients. (A) Amount of deletions at 888, 896, 902-bp did not show significant difference in terms of age. (B) Common deletion of 4977-bp was correlated

with age, and thus inversely correlated with the value of mean CTN (Pearson correlation coefficient = -0.195, p=0.019).

6. Relations between SCINEXA score and the amount of common deletion

With 73 patient samples from the face, correlation between SCINEXA score and the amount of common deletion was evaluated. The mean age of the subjects was 58.08 ± 21.65, and ranged from 6 to 93. As a result, SCINEXA score was positively correlated with the amount of common deletion in both intrinsic and extrinsic parts (Figure 9).



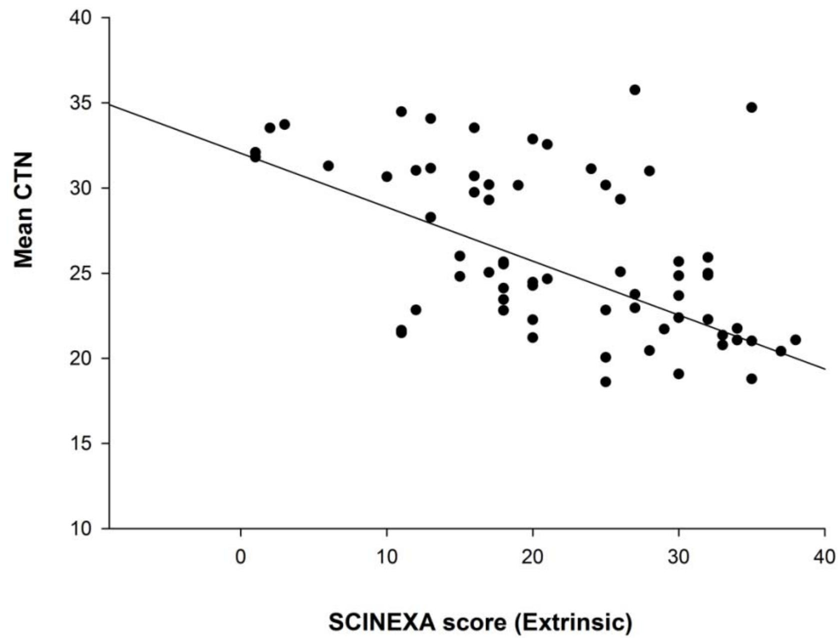
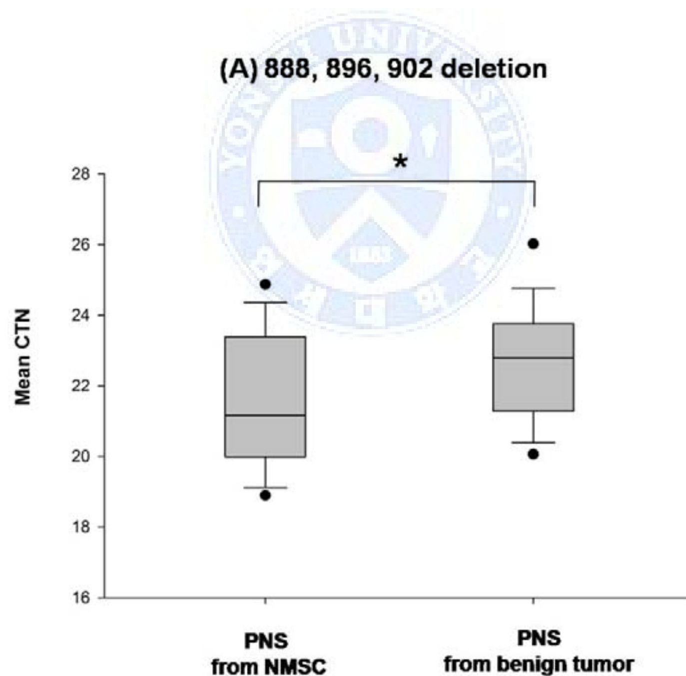


Figure 9. Correlation between SCINEXA score and the amount of common deletion. SCINEXA score was inversely correlated with significant decrease in CTN value in both intrinsic and extrinsic parts (Intrinsic score: Pearson correlation coefficient = - 0.447, p<0.001, Extrinsic score: Pearson correlation coefficient = - 0.415, p<0.001).

7. Comparison of mtDNA deletions from peri-lesional normal skin of NMSC and of benign tumor

In order to test the possibility of predicting the occurrence of NMSC from a deletion at a certain site of mtDNA, the amount of mtDNA deletion from the

peri-lesional normal skin of 76 NMSC patients and 68 benign tumor patients were compared. As a result, the amount of common deletions from NMSC and benign tumors were different with CTN values of 24.33±7.25 and 27.27±10.62, respectively, although not statistically significant due to large standard deviation (Independent sample t-tests, p=0.058). On the other hand, in case of the newly developed 888,896,902-bp deletion, it was 21.71±1.95 in peri-lesional normal skin of NMSC and 22.68±1.81 in that of benign tumors. Although the difference in means were not large, it was nevertheless significant statistically due to small standard deviation (Independent sample t-tests, p=0.002) (Figure 10).



* p<0.05, Independent sample t-tests

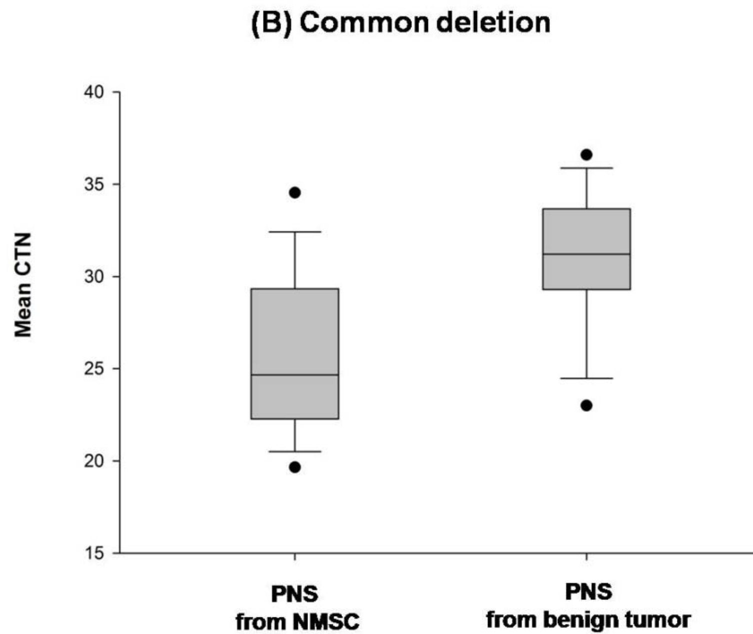


Figure 10. mtDNA deletions of peri-lesional normal skin (PNS) from NMSC and benign tumors. (A) The CTN value of deletion at 888, 896, 902-bp was significantly decreased at PNS of NMSC (p=0.002). (B) The value of common deletion at 4977-bp was not significantly different between the two lesions (p=0.058).

8. Multivariate analysis to calculate the risk of NMSC

Factors that may contribute to the prediction of NMSC occurrence were investigated using logistic regression analysis in respect to the subjects' sex and age, site of exposure (face, neck and extremities), the amount of common deletions and of 888, 896, 902-bp deletions. As a result, the odds ratio with

respect to increasing age was 1.104 (95% confidence interval: 1.066-1.143, $p<0.001$), and the odds ratio of the exposed sites compared to non-exposed sites was 9.300 (95% confidence interval: 3.001-28.819, $p<0.001$). There was no significant relation with respect to common deletions. However, in case of the amount of 888, 896, 902-bp deletion, the increase in the value of CTN was inversely correlated with the risk of NMSC (Odds ratio = 0.709, 95% confidence interval: 0.535-0.940, $p<0.05$).

Table 3. Multivariate analysis to calculate the risk of non-melanoma skin cancer

	OR (95% CI)	p-value
Age	1.104 (1.066-1.143)	$p<0.001$†
Sex	0.728 (0.259-2.047)	0.547
Exposure vs. non-exposure	9.300 (3.001-28.819)	$p<0.001$†
CTN values of common deletion	0.977 (0.918-1.039)	0.457
CTN values of new deletion	0.709 (0.535-0.940)	*$p < 0.05$

*$p < 0.05$, †$p < 0.001$, CI; confidence interval, OR; Odds ratio, CTN; Cycle threshold number, Statistical methods: logistic regression analysis

IV. DISCUSSION

Mitochondrial DNA (mtDNA) is a double-stranded closed circular molecule consisting of 16,569 nucleotides, and 100-10,000 copies exist in a single cell. The human mtDNA encodes 13 polypeptides, which are essential constituents of respiratory enzyme complexes, and 22transfer RNAs and two ribosomal RNAs that are required for protein synthesis in mitochondria.[18] The complete mtDNA sequence was determined in 1981,[19] and resequenced in 1999.[16] Mitochondrial DNA is exclusively maternally inherited, thus different mtDNA lineages cannot mix or recombine: the only way that the mtDNA sequence can change is by the sequential accumulation of mutations.[20]

Worth noting is the fact that mammalian mtDNA is more susceptible to oxidative damage compared with nuclear DNA. Since it is not associated with protective histones,[21] it is continually exposed to high levels of reactive oxygen species (ROS) generated by oxidative phosphorylation, and there is a limited capacity for mtDNA repair.[22,23] Fortunately, mtDNA forms heteroplasmy in a cell and mutated mtDNA is compensated for by wild-type mtDNA.[10] However, as the damage increases a threshold level for mutations is reached (50–60% for deletions and 60–90% for point mutations), impairment of the oxidative phosphorylation (OXPHOS) system and respiratory chain dysfunction can occur.[24]

These mtDNA mutations and rearrangements are currently under active research in relations not only to muscle and neurodegenerative diseases,[25,26] but cancers as well.[27,28] In particular, a large number of mtDNA mutations have been detected in a variety of cancers,[29,30] which are often found in cancerous tissue and thus have the potential to be a biomarker for cancer occurrence in the tissue, which has been published through 38 meta-analyses.[31]

In this study, the author sought to suggest a skin aging index that can predict the occurrence of NMSC using an analysis of mtDNA deletions. First, any difference in sequencing of NMSC from that of peri-lesional normal skin was assessed using mito-resequencing array. As a result, up to 18 to 37 single base alterations of mtDNA were found, although there was no difference between NMSC and normal skin lesions. On the other hand, the values marked "N" were different between the two groups. An "N" was a position where not more than 50% of sequences were detected, and this signifies that these are sites where potentially many copies of deleted mtDNA would be found. In SCC samples particularly, the "N" points were detected in proximity to 888, 896, 899, and 902-bp, and thus it was predicted that identifying the number of deleted mtDNA copies at these regions would be helpful in approximating the risk of SCC occurrence. In addition, it is considered that the problems on the mtDNA of 12rRNA may influence occurrence of SCC. Since 12s rRNA is also involved in metabolism and control of ROS, it can be inferred that a high

incidence of amino acid changing mutations in the 12s rRNA complex causes increased ROS production and subsequently increases mtDNA mutation and dysfunction, which creates conditions favoring tumor cell growth.

In multivariate analysis taking into account the patient age, sex, exposure site, it was found that the amount of new mtDNA deletions and occurrence of NMSC was related, given the odds ratio for the increase in CTN value and the risk of NMSC is 0.709. However, age and new deletion copy number were not found to be correlated. Although the amount of new mtDNA deletion cannot reflect the aging of skin, it would be helpful in approximating the incidence of NMSC. Incidence of SCC increases as life expectancy and amount of ultraviolet light exposure increase, and it is known that about 60% of SCC develops from actinic keratosis, which is a precancerous lesion.[32] However, the actual incidence of actinic keratosis being transformed into an invasive SCC is difficult to predict, and the reported figures vary from 0.025% to 20%.[33] Based on the results of this study, the risk of SCC can be predicted at second hand, and appropriate treatment of actinic keratosis can be selected among various field or local treatments.[34,35]

The amount of deleted mtDNA dependent on increasing age was significantly related to common deletion of 4977-bp. Also, with 73 skin samples from the face, correlation between SCINEXA score and amount of common deletion was found. 4977-bp 'common' deletion is one of the most

studied large scale deletions proposed as biomarkers of photoaging, and was shown that there was a significant increase between the incidences of high levels of the deletion in sun-exposed sites compared to sun-protected sites.[36] In addition, these findings have been confirmed in other groups of Caucasians.[37] Even though studies that were based on Asian subjects showed similar results, it might have limited use as an index of actual skin aging because the standard deviation for CTN value is large. In a recent study which evaluated the relationship between skin aging and actual age using deletions of 3895-bp, significant difference was found only when they were stratified into three groups (ages 18-35, 36-55, and over 56), and relationship with other skin aging index such SCINEXA score, or ethnic differences were not addressed.[11,13,38] mtDNA forms heteroplasmy and tends to compensate for damaged mtDNA, and there are reports about increasing age and the number of mtDNA copy numbers,[39] which suggests that mtDNA copy number increases as the age increases,[40-42] while others say that they are inversely related,[43-45] and still others say that there is no such relationship.[46-49] Therefore, while it is a valid statement that the copy number of deleted mtDNA is reflective of the degree of skin aging, it may have a limited use as an index of skin aging in clinical settings because the range of age distribution is rather large.

Our findings substantiate the rationale for exploring the mitochondrial genome as a biomarker for the early diagnosis of non-melanoma skin cancer.

In addition, a quantitative analysis suggests that large deletions of mtDNA such as common deletions are related to skin aging in Korean subjects also. However, it may have a limited use as an index of skin aging in clinical settings because the range of age distribution is rather large.

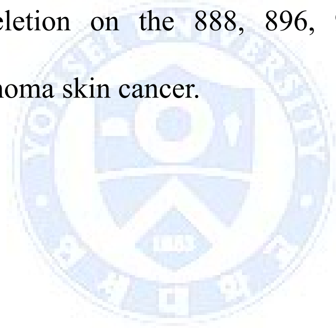
V. CONCLUSION

Using Mito-resequencing assay, the author sought to investigate the difference between mtDNA sequences in lesional and non-lesional areas of NMSC samples, and suggest a skin aging index which can predict the occurrence of NMSC in Korean patients by the quantitative analysis of specific mtDNA deletions using RT-PCR.

1. There was no difference in mtDNA alteration between the lesional and non-lesional areas of NMSC samples from the results of mito-resequencing assay.
2. On mito-resequencing assay, there were 28 and 11 "N" points, which were positions where not more than 50% of sequences were detected, in SCC samples and BCC samples, respectively. In SCC samples, the "N" points were detected in proximity to 888, 896, 899, and 902-bp.
3. mtDNA copy number containing new deletions (del888, del896, del902) were significantly higher in SCC lesions than peri-lesional normal skins.
4. The amount of new deletions was significantly higher in the peri-lesional normal skin of NMSC than that of benign tumor.
5. There was no significant correlation between increase in age and the amount of new deletions.

6. The amount of 4977-bp common deletion was significantly related with aging, although standard deviation was large.
7. In multivariate analysis taking into account the patient age, sex, and exposure site, it was found that the amount of new deletions and occurrence of skin cancer are related, given the odds ratio for the increase in CTN value and the risk of NMSC is 0.709.

Based on these results, large deletions of mtDNA such as common deletions are related to skin aging in Korean subjects also. However, it may have a limited use as an index of skin aging in clinical setting because the range of age distribution is rather large. In Korean patients, an analysis of mitochondrial DNA deletion on the 888, 896, 902-bp can predict the occurrence of non-melanoma skin cancer.



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ABSTRACT (IN KOREAN)

한국인의 피부암 발생을 예측할 수 있는 피부노화지표의 개발

<지도교수 정 기 양>

연세대학교 대학원 의학과

오 병 호

화장품, 미용시술 등의 항노화산업이 발전하면서 피부노화를 객관적으로 평가할 수 있는 지표의 필요성이 대두되고 있으나, 아직 표준화된 방법이 제시되지 않았다. 미토콘드리아는 세포내에서 활성산소의 생성과 조절을 담당하는 기관으로 인간의 노화과정과 밀접한 기관이다. 특히, 미토콘드리아 DNA 는 세포내에 100-10,000 개가 존재하는데, 손상발생시 복구되는 기전이 존재하지 않고 이형세포질성 (heteroplasmy) 으로 축적되는 성질을 가지므로 손상 후 결손이 발생한 양을 측정하면 노화정도를 계산할 수 있을 것으로 예상된다. 이에 본 연구에서는 특정 미토콘드리아 DNA 의 결손정도를 정량적으로 분석하여 피부노화와의 연관성을 확인하고 피부노화의 가장 큰 합병증인 피부암 발생을 예측할 수 있는 지표를 제시하고자 하였다.

먼저 미토콘드리아 리시퀀싱 분석 (Mito-resequencing assay)을 이용하여 피부암 병변부위와 비병변부위의 미토콘드리아 DNA 염기서열의 차이를 조사한 결과, 편평세포암에서 결손가능성이 높은 부위는 28개 부위, 기저세포암에서는 11개 부위가 관찰되었다. 이중

편평세포암 병변에서는 인접한 888, 896, 899, 902 base pair (bp) 부위가 결손 가능성이 높은 것으로 관측되어 이를 정량적으로 측정할 수 있는 프라이머 (primer)를 제작하였다. 그 결과, 888, 896, 902bp 부위의 결손을 가지고 있는 미토콘드리아 DNA의 양은 편평세포암 부위에서 주변의 정상피부에 비해 통계적으로 유의하게 증가하였다 (21.77±0.46 vs. 20.62±0.40, $p<0.001$). 또한, 68개의 양성종양 주변의 정상조직과 76개의 피부암주변의 정상조직을 비교한 결과, 피부암 주변의 정상조직에서 결손양이 증가한 것을 확인하였다 (22.68±1.81 vs. 21.71±1.95, $p=0.002$). 그러나, 888, 896, 902bp 부위의 결손양은 연령증가와는 유의한 연관성을 보이지 않아 노화지표로의 적용은 한계점을 나타냈다. 기존의 4977bp의 결손이 연령증가에 따라 유의한 상관관계를 보여 피부노화와의 연관성을 보였으나, 상관계수가 0.195로 연령대별 표준편차가 큰 경향을 보여 임상에서의 적용은 한계가 있을 것으로 생각된다. 피부암 발생의 예측인자를 알아보기 위해 다변량 분석을 시행한 결과에서는 888, 896, 902bp 부위의 결손양이 적을수록 교차비 0.709만큼 피부암 발생이 줄어든다는 것을 확인하였다.

결론적으로 한국인에서 미토콘드리아 DNA 의 888, 896, 902bp 부위의 결손양을 측정하여 피부암 발생을 예측할 수 있는 지표로 활용할 수 있다.

핵심어: 미토콘드리아 DNA, 피부노화지표, 피부암, 미토콘드리아 DNA 결손분석, 염기서열분석