A4. Effects of Ultraviolet Ray Increase on Human Health

A-4.1 Molecular Epidemiological Study using Biological Markers like Point Mutation on the Relationship between Exposure to Ultraviolet Ray and Skin Cancer.

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Total Budget for FY 1990-FY 1992 37,459 Yen

Key Words UV, skin cancer, solar keratosis, p53, pyrimidine dimer, epidemiology

Abstract

Analyses of trends in mortality from skin cancer in Japan and standardized mortality rates by prefecture (SMR) revealed decreased cumulative mortality rates of non-melanocytic skin cancer in both males and females in the last 30 years. SMR, however, showed higher rates in the southern part of Japan such as Okinawa, Kagoshima, Kochi, etc. These suggested the relationship between UV exposure and skin cancer.

To find a good biomarker for premalignant state of skin cancer being related to the UV exposure, p53 aberrations in both DNA sequence and immunostaining for p53 protein was examined. Aberrations of the p53 gene in 29 solar keratosis (SKs) and 5 squamous cell carcinomas (SCCs) were examined by single-strand conformation polymorphism analysis of polymerase chain reaction products. In a series of Japanese patients, 8/29 (28%) of SKs and 2/5 (40%) SCCs of the skin contains structural abnormalities of the p53 gene. We clarified that aberrations of the p53 gene were caused in SKs of early dysplasia.

Introduction

Prevalence, incidence and/or mortality rate of non-melanocytic skin cancer as well as malignant melanoma among Japanese belong to the lowest group in the world (1). Decreased cumulative mortality rates of non-melanocytic skin cancer in both males and females in Japan diminishes the size of problem (2). Incidence rate of skin cancer, however, is obscure, because it is obtained only from a few prefectures, such as Nagasaki, Hiroshima and Miyagi. From these limited sources non-melanocytic skin caner seems to be increasing. It is important to know whether or not the incidence of non-melanocytic skin cancer and malignant melanoma will increase as a result of increasing irradiation of ultreaviolet ray
(UV) which has been caused by the enlargement of ozone holes (3).

Study groups for the Effects of Ultraviolet Ray Increase on Human Health were established by the Environmental Agency in 1990 under the Global Environment Research Program to clarify the

adverse effects of UV for Japanese (4).
The purpose of this research project is to clarify the effect of exposure to ultraviolet ray on the skin cancer in Japan.

Research Object

(1) Time trends in skin cancer mortality and standardized mortality ratio were calculated. Time trends in out-patient clinic in the university hospitals from selected areas, such as Hokkaido (lattitude 43 degree), Saitama (36 degree), Fukuoka (33.5 degree), Kagoshima (31.5 degree) and Okinawa (26 degree), were analysed. Trends of skin cancer in these areas and histological review
were planned.

(2) Biomarkers for early lesion due to UV exposure were detected. Pyrimidine dimers, accumulation of p53 gene product, and p53 gene alterations were main focus.

(3) Japanese immigrants in Belen and Sao Paulo, Brazil are also planned to be studied.

Research Methods

(1) Time trends in skin cancer mortality and standardized mortality ratio were calculated from the vital statistics provided by the Ministry of Health and Welfare. Dermatologists in the university hospitals in the selected areas for the study are Hokkaido (lattitude 43 degree), Saitama (36 degree), Fukuoka (33.5 degree), Kagoshima (31.5 degree) and Okinawa (26 degree), were requested to collect data during the last 10 years. Trends of skin cancer in these areas and histological review were performed.

(2) Biomarkers for early lesion due to UV exposure were detected. Pyrimidine dimers, accumulation of p53 gene product, and p53 gene alterations were main focus. Surgical specimens were fixed with the AMeX method or embedded in OCT compound to perform tissue sections, and subsequently stored at -80C until use.
DNA samples were subjected to the polymerase chain reaction in the mixture described below using two appropriate oligonucleotides as primers. The oligonucleotide primers for PCR amplification of all p53 exons were designed as described below.

The PCR reaction solution contained 1.25 mM dATP/1.25 mM dGTP/1.25 mM dCTP/1.25 mM dTTP/0.75-1.0 mM MgCl2/10 mM Tris-HCl pH 8.3/0.1% gelatine/10 5M one set of primers/0.1 5g of DNA/0.2 51 of [32P]dCTP (3000 5Ci/mmol, 10 mCi/51, Amersham) /0.5unit of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CA) and 12 51 mineral oil. Electrophoresis was performed at 30 W for 2 to 4 h with cooling by a fan. The gel was dried on filter paper and exposed to X-ray film at -80!C for 4 to 12 h using an intensifying screen.

Direct DNA Sequencing. DNA fragments showing mobility shift by PCR-SSCP analysis were eluted from polyacrylamide gel, and were amplified by 30 cycles of the symmetric PCR under the same conditions as describe above. The reaction mixture was diluted and deionized in a Centricon 30 microconcentrator. After the amplified DNAs had been annealed with the 5'-labeled primers.

Nucleotide sequences were determined using a dsDNA Cycle Sequencing System.

Immunohistochemical staining was performed on AMeX sections and frozen sections using an avidin-biotinylperoxidase complex (ABC) method. The monoclonal antibody to p53 protein, pAb 1801, was obtained from Oncogene Science Inc.

(3) Japanese immigrants in Sao Paulo, Brazil are also planned to be studied. The census data were used for this purpose.

Results

(1) Mortality and incidence of skin cancer in Japan

Number of deaths from skin cancer between 1980-1990 was 3,822 in males and 3,207 in females. Standardized mortality ratio (SMR) by prefecture showed higher value in the southern part of Japan, such as Miyazaki, Kagoshima, Kochi, Chiba, etc. (6,7). Japan is a very long arc-like country, covering from 43 degree lattitude at Sapporo to 25 degree lattitude at Okinawa. Difference between highest and lowest SMR by prefecture was 1.8 fold in males and 2.0 in females. The high SMR areas seemed to be a sunny area. On the contrary, SMR of malignant melanoma did not show such regional cluster, although southern part belong to the high SMR area.

Mortality of skin cancer (ICD 172 and 173) decreased to one third during the last 30 years. Cumulative incidence rates of non-melanocytic cancer until age 74 were 0.1-0.7 in males and 0.09-0.39 in females, and those of melanoma were 0.02-0.07 in males and 0.02-0.08 in females in Japan.

(2) Case collection in the Department of Dermatology in the above areas suggested more frequent solar keratosis and skin cancer in the above region, but the exposure to sun beam seemed to be attributable in only one tenth of squamous cell carcinoma cases. For example, cases in the out-patient clinic in Kagoshima University in 1990 are as follows:

Diagnosis	Exposed site	Non-exposed	Both
Solar keratosis	14	0	
Bowen disease	1	3	1
Squamous cell ca	14	5	
Basal cell ca	18	3	
Seborrheic keratosis	38	23	4
Keratoacanthoma	2		

(3)p53 gene alterations

A difference in the frequency of p53 mutations between tumor types since 28% (8/29) of the solar keratosis presented compared with 40% (2/5) of the SCCs (Table 2). These mutations were detected in exons 4, 5, 6, 7, 8, and 10. Most of the mutations were single-nucleotide missense mutations. Further, in cases SK5, SCC3, SK27 and SK33, which showed nonsense mutation, double-nucleotide substitution, 4 base deletions and 5 base deletions respectively (Table.1). In the eight cases showing substitutions, five transitions (four C to T and one T to C), three transversions (one T to G and one C to A) and one double-nucleotide

substitution (CC to GT), were found. Five C to T substitutions (involved a double-nucleotide substitution), that all of these were existed on the CpG methylation site (Table. 2).

The positivity of nuclear staining for p53 protein was significantly correlated with the presence of mutation of p53 gene ($P<0.01$), but number of gene alteraion to stop codon was not negligible.

Table 1 *Aberrations of p53 gene and p53 protein in epithelial skin tumors*

Case	Histological	Tumor site	IHC staining	Aberration of p53 gene	p53 alteration Exon	Codon	Base change	Amino acid
Solar keratosis								
SK5	atrophic	cheek	+	−				
SK40	bowenoid	head	+	−				
SK19	bowenoid	cheek	+	+	4	109	TTC - TCC :T-C	Phe - Ser
SK18	atrophic	cheek	+	+	4	110	cC*GT - cTGT :C-T	Arg - Cys
SK33	bowenoid	nose	−	+	6	194	CTTAICCG - CTG	5base deletions
SK25	atrophic	temple	−	+	6	215	AGT - AGG :T-G	Ser - Arg
SK7	bowenoid	chest	+	+	7	248	cC*GG - cTGG :C-T	Arg - Trp
SK27	bowenoid	temple	−	+	8	271	GAGGTGC - GGC	4base deletions
SK8	bowenoid	chest	+	+	8	278	tCCT - tACT :C-A	Pro - Thr
SK4	bowenoid	cheek	−	+	10	342	cC*GA - cTGA :C-T	Arg - Stop
Squamous cell carcinoma								
SCC1		lower lip	+	−				
SCC3		cheek	+	+	7	247-248	AAC C*GG - AAG TGG :CC-GT	Asn Arg - Lys Trp
SCC4		head	+	+	7	248	acC*GG - acTGG :C-T	Arg - Trp

Discussion

(1) ICD cord 173 includes both squamous cell carcinoma and basal cell carcinoma, so the registration rate of these lesions is quite different according to the cancer registry. In Japan, cancer registries in Hiroshima and Nagasaki can provide satisfactory data as for the skin tumors. Most basal cell carcinoma of Japanese looks less malignant compared to the Caucasians, so the dermatologists would not report them to the cancer registry. Comparing these incidence rates with that of Japanese immigrants in Brazil, the incidence became almost five fold in Sao Paulo. Age-specific incidence curve of skin cancer except for melanoma among Brazilians and Japanese Brazilian showed a quite large difference between the two population. The incidence should be influenced by the strong sunlight in Sao Paulo. High SMR in southern part of Japan supported the finding that the strong UV may cause skin cancer in these region.

(2) 88% of SKs contained mutation spectrum for UV specific at dipyrimidine site. Thus, we could understand that UV exposure is playing a role for p53 gene alterations in SKs. Methylation of CpGs in normal tissues might increase the probability of mutations at such sites because of ability of 5-methylcytosine to undergo deamination, resulting in a thymine. The p53 gene mutation of UV induced skin cancer at CG dinucleotides suggests that they may be due to the 106-fold acceleration of cytosine deamination rate by cyclobutane dimers, occurring at 5-methylcytosine. Cases of nonsense mutants can not accumulate by p53 protein, suggesting that the lack of staining was not simply caused by loss of specific epitopes. Existence of skin-cancer-hotspot mutate to stop codon may be playing a role for low positive incidence by immunostaining of p53 protein. The positivity of nuclear staining for p53 protein was significantly correlated with the presence of mutation of p53 gene, although mutation to

stop codon and deletions were excluded from.

Immunohistochemical staining for p53 protein were positive in four out of five cases with amino acid substitution, resulting in a positivity of 80%. Comparing two methods, immunohistochemical staining and PCR-SSCP, highly correlated and statistically significant results were obtained. Thus, the use of immunohistochemical staining might be a convenient and an useful tool for screening the p53 missense mutations.

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