นิพนธ์ต้นฉบับ

Genetic instability during early stage of tumorigenesis predicts poor prognosis in p53 overexpressed head and neck cancer.

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Background: *Alterations of the p53 gene or protein have been known to be the most common abnormality of tumor suppressor genes identified in human cancers. Loss of p53 function has been believed to be one of factors resulting in genetic instability and poor prognosis.*

Objective: *We studied the role of p53 overexpression and genetic instability in p53 overexpressed head and neck squamous cell carcinomas (HNSCC) with and without development of recurrence of primary tumor (RPT) and/or second primary tumors (SPT).*

Methods: *We examined the genetic instability of chromosome 9 and 17 in fourty two p53 overexpressed HNSCC with and without recurrence and/or second primary tumors. P53 overexpression was determined by immunohistochemistry using anti-p53 mouse monoclonal antibodyDO7 and the genetic instability was studied in terms of the polysomy index (PI) by non-isotopic chromosome in situ hybridization using chromosomes 9 and 17 probes.*

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Results: *We found nonstatistic significant difference between genetic instability in these two groups. PI for chromosome 9 at the normal adjacent to malignant lesions, ANL was 1.59 ± 1.49 vs 0.36 ± 0.31 (p = 0.0705), at hyperplasia, HYP was 4.05 ± 3.69 vs 2.26 ± 1.11 (p = 0.1807), at dysplasia, DYP was 6.82 ± 5.19 vs 6.60 ± 5.30 (p = 0.8981) and at squamous cell carcinomas, SCC was 16.85 ± 9.76 vs 18.80 ± 11.84 (p = 0.5784) and PI for chromosome 17 at ANL was 2.00 ± 1.97 VS 0.49 ± 0.38 (p = 0.0654), at hyperplasia, HYP was 3.51 ± 2.50 vs 3.31 ± 3.15 (p = 0.8793), at dysplasia, DYP was 9.03 ± 5.65 vs 9.58 ± 7.11 (p = 0.7948) and at squamous cell carcinomas, SCC was 20.70 ± 10.77 vs 21.39 ± 14.32 (p = 0.8635) respectively.*

Conclusion: *We concluded that p53 overexpression and genetic instability occured in the early stage (from normal adjacent to malignant lesions) during tumorigenesis of the HNSCC. The genetic instabilty in p53 overexpressed HNSCC probably associated with more aggressive disease and thus a poor prognosis. Even though we could not demonstrate the significance by means of statistics, we believe that this could be used as the prognostic marker for the disease outcome.*

Key words: *P53 overexpression, Genetic instability, Poor prognosis, Head and neck tumorigenesis.*

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นวพรรณ จารุรักษ์, นรินทร์ วรวุฒิ. ความไม่มีเสถียรภาพของยีนในระยะแรกของการเกิดโรคมะเร็งใช้ในการทำนายโรคที่ไม่ดีในโรคมะเร็งศีรษะและคอที่มีความผิดปกติของโปรตีน p53. จุฬาลงกรณ์เวชสาร 2539 ก.ย.;40(9): 725-36

เหตุผลของการวิจัย: *การเปลี่ยนแปลงของยีน P53 หรือการสูญเสียหน้าที่ของโปรตีน P53 เป็นการเปลี่ยนแปลงที่พบว่าเกิดขึ้นมากที่สุดในโรคมะเร็ง การที่โปรตีน P53 ไม่สามารถทำหน้าที่ได้ตามปกติเชื่อว่าเป็นสาเหตุหนึ่งของการเกิดความไม่มีเสถียรภาพของยีนอื่นและการทำนายโรคที่ไม่ดี*

วัตถุประสงค์: *เพื่อศึกษาบทบาทของการเกิดความผิดปกติของโปรตีน P53 และการเกิดความไม่มีเสถียรภาพของยีนในโรคมะเร็งของศีรษะและคอชนิดสแควร์มัสเซลล์ ในกลุ่มที่เกิดการกลับเป็นใหม่ของมะเร็งปฐมภูมิ (recurrence of primary tumor, RPT) และ/หรือเกิดมะเร็งปฐมภูมิครั้งที่สอง (second primary tumors, SPT) และในกลุ่มที่ไม่เกิดการกลับเป็นใหม่ของมะเร็งปฐมภูมิ และ/หรือ มะเร็งปฐมภูมิครั้งที่สอง*

วิธีการ: *คณะผู้วิจัยทำการศึกษาความไม่มีเสถียรภาพของยีนของโครโมโซม 9 และ 17 ในผู้ป่วยจำนวนทั้งสิ้น 42 รายที่เกิดความผิดปกติของโปรตีน P53 จากผู้ป่วยโรคมะเร็งศีรษะและคอจำนวน 60 ราย ด้วยวิธีอกัมมันตรังสีอินไซตูไฮบริไดเซชั่น (non-isotopic in situhybridization, NISH) ในเทอมของ ดัชนีโพลีโซมี (polysomy index, PI)*

ผลการศึกษา: *คณะผู้วิจัยพบความแตกต่างอย่างไม่มีนัยสำคัญทางสถิติ ($p>0.05$) ของ PI 9 และ 17 ในกลุ่มทั้งสองโดย PI 9 ในเนื้อเยื่อเซลล์รูปร่างปกติ (normal adjacent to malignant lesions, ANL) เท่ากับ 1.59 ± 1.49, ในเนื้อเยื่อที่หนาตัวขึ้น (hyperplasia, HYP) เท่ากับ 4.05 ± 3.69, ในเนื้อเยื่อที่เซลล์มีรูปร่างผิดปกติ (dysplasia, DYP) เท่ากับ 6.82 ± 5.19, และในเนื้อเยื่อที่เซลล์ที่เปลี่ยนรูปร่างเป็นเซลล์มะเร็ง (squamous cell carcinomas, SCC) เท่ากับ 16.85 ± 9.76 ในกลุ่มแรก เปรียบเทียบกับ ANL = 0.36 ± 0.31, HYP = 2.26 ± 1.11, DYP = 6.60 ± 5.30, และ SCC = 18.80 ± 11.84 ในกลุ่มหลัง ส่วน PI 17 เท่ากับ ANL = 2.00 ± 1.97, HYP = 3.51 ± 2.50, DYP = 9.03 ± 5.65, และ SCC = 20.70 ± 10.77 ในกลุ่มแรก เปรียบเทียบกับ ANL = 0.49 ± 0.38, HYP = 3.31 ± 3.15, DYP = 9.58 ± 7.11, และ SCC = 21.39 ± 14.32 ในกลุ่มหลังตามลำดับ*

สรุป: *คณะผู้วิจัยสรุปว่าการเกิดความผิดปกติของโปรตีน P53 และการเกิดความไม่มีเสถียรภาพของยีนเกิดขึ้นตั้งแต่ในระยะต้น คือ ANL ในระหว่างขบวนการการเกิดเป็นโรคมะเร็งศีรษะและคอ และก่อให้เกิดโรคมะเร็งศีรษะและคอที่มีความรุนแรงและมีการทำนายโรคที่ไม่ดี อย่างไรก็ตามแม้ว่าในการวิจัยนี้ไม่สามารถแสดงให้เห็นความสัมพันธ์หรือความแตกต่างได้อย่างชัดเจนและมีนัยสำคัญจากข้อมูลทางสถิติ ผู้วิจัยเชื่อว่าการตรวจหาความไม่มีเสถียรภาพของยีนในระยะต้นนี้อาจนำมาใช้เป็นตัวบ่งชี้หรือทำนายโรคได้*

The prognosis of patients suffering from head and neck squamous cell carcinomas (HNSCC) is adversely affected by occurrence of recurrence of primary tumor (RPT) and second primary tumors (SPT).[1,2] The recent studies concerning the loss of p53 gene function resulting in the p53 protein overexpression have been shown to affect cell cycle control and may lead to genetic instability.[3-5] Furthemore, the p53 overexpression has been reported to be associated with poor prognostic outcome as a consequence of RPT and SPT.[6-8]

This study attempted to determine the role of p53 overexpression and genetic instability during tumorigenesis of HNSCC with and without RPT and/or SPT.

Materials and Methods

Tissue Preparation. Sixty formalin-fixed, paraffin-embedded tissues of tumor specimens were obtained from patients with HNSCC who were treated surgically between 1980 and 1991. Some specimens contained not only tumors but also normal adjacent epithelial, and premalignant lesions. Fourty two of the 60 samples demonstrated immunocytochemical reactivity of the p53 expression; 15 exhibited normal adjacent to maliganant lesions (ANL); 19 hyperplasia (HYP); 38 dysplasia (DYP); and 42 squamous cell carcinomas (SCC). Eighteen specimens were negative for the p53 expression. This group consisted of 34 ANL, 31 HYP, 26 DYP, and 18 SCC. Sections 4 μm thick were mounted on aminoalkylsilance-coated slides (Histology Control Systems, Glen Head, NY). The slides were stored at room temperature.

Probe. A biotinylated classical satellite chromosome 9 (D9Z1) probe specific for the pericentric heterochromatin of human chromosome 9 and a biotinylated alpha satellite chromosome 17 (D17Z1) probe specific for centromere of human chromosome 17 (Oncor, Inc., Gaitherburg, MD) were used for in situ hybridization (ISH). An anti-p53 murine monoclonal antibody (clone D07; Biogenex Inc., San Ramon, CA) was chosen for immunohistochemistry (IHC).

Immunohistrochemistry (IHC). The IHC procedure was performed as previouly[9,10] described. In brief, after deparaffinization and blocking of endogenous peroxidase, the anti-p53 mouse monoclonal antibody-D07 was incubated at 37°C for 2 hours. The slides were then incubated with a biotinylated horse antimouse secondary antibody (Vector Labs., Burlingame, CA) and were visualized with an avidin-biotin immunoperoxidaes system (Vector) using 0.1% diaminobezidine as a chromogen and counterstain with 4% methyl green. Only nuclear localization of immunoreactivity was evaluated. The fraction of positively stained nuclei (%) was scored in each defined histological area. A cell block section of paraffinembedded A431 cells which express a mutant p53 gene (CGT to CAT at codon 273), was attached to each slide that was prepared for IHC and used as a positive control, and lymphocytes on each of the sections were used as negative controls.

Chromosome In Situ Hybridization (CISH). Only fourty two p53 overexpressed samples were chosen for CISH study. The CISH procedure was performed as previously described [11] with slight modification. Briefly, the specimens were deparaffinized after 65°C overnight incubation and

were treated with 1mg/mL RNase in 2XSSC (1XSSC = 0.15M NaCl, 15mM Na_3citrate.$2H_2O$). Each specimen was digested with 0.4% pepsin (Sigma, St. Louis, MO) in 0.2N HCl. The optimal digestion for each slide was determined under a light microscopic examination. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. The hybridization solution contained 60 % formamide in 2XSSC, 5% dextran sulfate, 1mg/mL salmon sperm DNA, and 0.8ng/uL biotinylated DNA probes.Twenty to 30 µL of the hybridization solution were applied varying according to the size of each section. The probe and target DNA were denatured together at 95°C, 4 min for chromosome 17 and 96°C, 6 min for chromosome 9 and then incubated overnight at 37°C. The slides were treated in 50% formamide (pH7.0), and 0.1XSSC (pH7.0). Histochemical detection of the probe was performed by immunoperoxidase staining. The slides were incubated for 30 min at 37°C with avidin and biotinylated antiavid in D (Vector Labs., Inc., Burlingame, CA) in 3% bovine serum albumin in 1XPBS (1XPBS = 137mM NaCl, 2.7mM KCl, 4.5mM Na_2HPO_4.7H2O, 1.4mM KH_2PO4, 0.16mM $CaCl_2$, 0.5mM Mg Cl_2.$6H_2O$), and with avidin-biotin-peroxidase complex solution (Vectastain ABC kit; Vector Labs., Inc.) Visualization was achieved with 50mL 1XPBS solution containing 50 mg diaminobezidine tetrahydrochloride (Sigma), 35mg of $NiCl_2$, and 10uL of 30% H_2O_2. The slides were then counterstained with Giemsa stain (0.02%), mounted in Eukitt (Calibrated Instruments, Inc., Hawthorne, NY), and examined under a light microscope. Areas for analysis were selected by comparing the hybridized slides to a corresponding hematoxylin-eosin-stained adjacent section. At least 200 nuclei were scored in each defined histological area according to the previously described criteria.[12] A polysomy index (PI) is defined as the percentage of cells exhibiting 3 or more copies of a chromosome. The lymphocytes on the same sections served as internal controls.

Statistical Analysis. Statistical analyses were performed using the unpaired Student's t test for testing the significance. $P \leq 0.05$ was considered statistically significant.

Results

We studied sixty specimens for 49 ANL, 50 HYP, 64 DYP, and 60 SCC. Fourty two of these 60 samples expressed p53 protein detected by anti-p53 monoclonal antibody-DO7, were divided into two groups, the group with and without RPT and/or SPT; consisted of 15 ANL, 19 HYP, 38 DYP, and 42 SCC. Eighteen of these 60 were not demonstrated p53 expression by anti-p53 monoclonal antibody-DO7.

As shown in Table 1, we chose 42 patients with p53 overexpressed HNSCC and divided the patients into two groups, the first group with and the second group without RPT and/or SPT. IHC was performed using anti-p53 monoclonal antibodies on paraffin sections. These 42 cases consisted of 15 ANL, 19 HYP, 38 DYP, and 42 SCC. The specimens of the group who developed RPT and/or SPT consisted of 9 ANL, 10 HYP, 18 DYP, and 13 SCC, and the group without RPT and/or SPT consisted of 6 ANL, 9 HYP, 20 DYP, and 29 SCC. The specimens were obtained from different head and neck sites. These were the oral cavity (30 patients), oropharynx

(9 patients), hypopharynx (4 patients), larynx (13 patients) and others (4 patients). Forty six patients were males and 14 were females.The median age was 60 years old (range 37-86).

Table 1. Patient characteristics and histology.

Characteristics	Total	P53-expressed group	P53 non-expressed group
- Patient Number	60	42	18
- Gender			
Female	14	9	13
Male	46	33	5
- Age (year)			
Median	60	60	60
Range	37-86	37-86	48-76
- Site			
Oral Cavity	30	21	9
Oropharynx	9	6	3
Hypopharynx	4	3	1
Larynx	13	8	5
Other	4	4	0
- Histology			
Normal (adjacent to tumor)	49	15	34
Hyperplasia	50	19	31
Dysplasia	64	38	26
Squamous cell carcinoma	60	42	18

To determine the role of p53 overexpression in genetic instability during tumorigenesis of HNSCC with and without RPT and/or SPT, we analyzed the chromo- some changes in terms of PI in the 42 p53 expressed specimens according to the histologic areas. In both chromosome 9 and 17 we found nonstatistic significant difference between genetic instability in these two groups. PI for chromosome 9 at the normal adjacent to malignant lesions, ANL was 1.59 ±1.49 vs 0.36 ± 0.31 (p = 0.0705), at hyperplasia, HYP was 4.05 ± 3.69 vs 2.26 ± 1.11 (p = 0.1807), at dysplasia, DYP was 6.82 ± 5.19 vs 6.60 ± 5.30 (p = 0.8981) and at squamous cell carcinomas, SCC was16.85 ± 9.76 vs 18.80 ± 11.84 (p=0.5784) and PI for chromosome 17 at ANL was 2.00 ± 1.97 VS 0.49 ± 0.38 (p = 0.0654), at

hyperplasia, HYP was 3.51 ± 2.50 vs 3.31 ± 3.15 (p = 0.8793), at dysplasia, DYP was 9.03 ± 5.65 vs 9.58 ± 7.11 (p = 0.7948) and at squamous cell carcinomas, SCC was 20.70 ± 10.77 vs 21.39 ± 14.32 (p = 0.8635) respectively (Table 2 and 3). However, the results showed a higher trend in the group with RPT and/or SPT over the group without RPT and/or SPT on the premalignant lesions whereas such trend was not noticeable in the malignant lesions.

Table 2. The influence of p53 overexpression on genetic instability in term of PI for chromosome 9 over the group with and without recurrence and or SPT during head and neck tumorigenesis.

Histopathology (number)	PI 9 (± SD)		P value
	no REC,no SPT	REC and/or SPT	
ANL (n=15)	0.36 (± 0.31) (n=6)	1.59 (± 1.49) (n=9)	0.0705
HYP (n=19)	2.26 (± 1.11) (n=9)	4.05 (± 3.69) (n=10)	0.1807
DYP (n=38)	6.60 (± 5.30) (n=20)	6.82 (± 5.19) (n=18)	0.8981
SCC (n=42)	18.80 (± 11.84) (n=13)	16.85 (± 9.76) (n=29)	0.5784

SD,standard deviation
REC,recurrence
SPT,secound primary tumors
PI,polysomy index

ANL,normal adjacent to malignant lesions
HYP,hyperplasia
DYP,dysplasia
SCC,squamous cell carcinomas

Table 3. The influence of p53 overexpression on genetic instability in term of PI for chromosome 17 over the group with and without RPT and/or SPT during head and neck tumorigenesis.

Histopathology (number)	PI 17 (± SD)		P value
	no REC,no SPT	REC and/or SPT	
ANL (n=15)	0.49 (± 0.38) (n=6)	2.00 (± 1.79) (n=9)	0.0654
HYP (n=19)	3.31 (± 3.15) (n=9)	3.51 (± 2.50) (n=10)	0.8793
DYP (n=38)	9.58 (± 7.11) (n=20)	9.03 (± 5.65) (n=18)	0.7948
SCC (n=42)	21.39 (± 14.32) (n=13)	20.70 (± 10.77) (n=29)	0.8635

SD,standard deviation
REC,recurrence
SPT,secound primary tumors
PI,polysomy index
ANL,normal adjacent to malignant lesions
HYP,hyperplasia
DYP,dysplasia
SCC,squamous cell carcinomas

We also studied the genetic instability in term of PI for both chromosomes 9 and 17 on the p53 non-expressed specimens during tumorigenesis of HNSCC with and without RPT and/or SPT (data not shown). Our results demonstrated the higher trend of increasing genetic instability in terms of PI for both chromosomes 9 and 17 on premalignant and malignant regions for the specimens with RPT and/or SPT over the group without RPT and/or SPT, but without statistical significance.

Furthermore, we determined the p53 overexpression in both groups with and without RPT and/or SPT. The p53 expression was higher in the group with RPT and/or SPT over the group without RPT and/or SPT, but no statistic significance could be demonstrated (data not shown).

Discussion

The early presence and character of stepwise accumulation of genetic instability[12] and p53 overexpression[13] that progressed increasingly from the ANL to HYP to DYP, and to head and neck cancer, suggested a potential role of genetic instability and p53 overexpression during multistep tumorigenesis. We determined the role of p53 overexpression on genetic instability in p53 overexpressed HNSCC with and without development of RPT and/or SPT. Our results demonstrated the higher trend of genetic instability in the group with and RPT and/or SPT over the group without RPT and/ or SPT in both p53 non-expressed and expressed specimens. These results supported the previous findings that p53 overexpression is probably associated with a more aggressive disease and poor prognosis,[6,8] and the influence of p53 overexpression on the genetic instability.[3,5] Cancer is a multistep process which is initiated by one or more hits to the genome, usually in the form of a mutation in growth-controlling genes, namely oncogenes or tumor suppressor genes, resulting in a certain level of relaxation of cellular growth controls. Cells in which proliferative controls are relaxed or uncoupled from appropriate regulatory control as a result of one or more primary hits apparently have a higher probability for the acquisition of secondary genomic alterations. The predominant view of initiation is that the concomitant gain of growth-promoting oncogene functions and the loss of negative regulators (ie, tumor suppressors) acts to propel the cell toward increasingly aberrant cell-cycle control at the molecular level and an increasingly malignant state at the phenotypic level. Secondary genomic alterations include a wide array of chromosomal aberrations such as aneuploidy, gene amplifications, translocations, and mutations that may in turn lead to activation of additional cellular oncogenes and loss of additional tumor suppressors.[5] The attainment of a certain number of primary plus secondary alterations results in tumor promotion and progression. These findings suggest that implication for carcinogenesis is that when p53 function is lost, the cell lacking p53 function become genetically unstable and thus predisposed to gross genomic alterations such as gene amplifications, aneuploidy, translocations, deletions,and mutations. These are not unexpected because the p53 tumor-suppressor gene fits the expectation, being altered in the vast majority of human cancers. The p53 protein acts as a molecular switch that activates a cell-cycle checkpoint in the G1 phase of the cell cycle.[14] When p53 is activated, it transactivates a whole battery of downstream effector genes, whose products are themselves involved in negative growth control. The likely purpose of this cell-cycle checkpoint is to provide the cell with a time window for repair of DNA damage prior to S-phase entry. The loss of p53 function thus creates conditions favorable for genetic instability. The evidence of the genetic instability in term of PI of both chromosome 9 and 17 and the presence of p53 overexpression at the early stage, ANL lesion, suggests that p53 overexpression may enhance genetic instabilty in the early stage (from aggressive normal adjacent to maliganant lesions) during tumorigenesis of HNSCC to activate more aggressive disease and thus a poor prognosis. The genetic instability status in the

early stage of p53 overexpressed HNSCC may be used as a prognosis marker for monitoring of the disease outcome. Furthermore, in the p53 non-expressed specimens, there was a higher trend of genetic instablity in the group with RPT and/or SPT over the group without RPT and/or SPT, suggesting that additional molecular pathways may be involved in the genetic instability and tumorigenesis.

These results suggest that p53 over expression and genetic instability occur early on the normal adjacent toward malignancies and probable activates more aggressive disease and thus a poor prognosis. However, genetic instability may be activated by p53 or other pathways which remain to be elucidated.

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