HER-2/neu Gene Amplification and Expression in Nasopharyngeal Carcinoma and Clinical Significance

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Abstract **Purpose** To study HER-2/neu gene Amplification and Expression in Nasopharyngeal Carcinoma (NPC) and clinical significance. **Methods** HER-2/neu Gene Amplification and Expression in NPC tissues were detected with fluorescence in situ hybridization (FISH, Vysis Path Vysion™ kit) and immounhistochemistry (IHC, DAKO Herceptin Test™ kit); NPC cell lines were tested by reverse transcription polymerase chain reaction (RT-PCR) and FISH and IHC to explore the mechanism of HER-2/neu Gene overexpression. **Results** No HER-2/neu gene amplification but gene overexpression was detected in NPC. HER-2/neu overexpression was caused by mRNA overexpression. **Conclusion** HER-2/neu gene has not been amplified, but overexpressed, HER-2/neu gene overexpression did not show prognostic significance in NPC.

Key Words Nasopharyngeal Carcinoma; HER-2/neu gene; gene amplification; gene expression

HER-2/neu gene appears to be diploid and deficient expression or low expression in normal cells, but amplification and overexpression have been identified in many human tumors. The gene amplification and overexpression may be associated with tumor origin and prognosis. Many studys of HER-2/neu gene expression in nasopharygeal cancer (NPC) had been reported[1], only few research about HER-2/neu amplication were performed. Based on the situation, in this experiment NPC paraffin samples were examined with fluorescence in situ hybridization (FISH) and immounhistochemistry (IHC) at the same time, to study the status of HER-2/neu gene amplification and expression and prognostic value in NPC; in addition, NPC cell lines were tested by reverse transcription polymerase chain reaction (RT-PCR) and FISH and IHC to study HER-2/neu gene amplification and expression so as to explore the mechanism of overexpression.

MATERIAL AND METHODS

Objects

40 nasopharyngeal carcinoma samples were collected from the patients who had been treated in the Cancer Hospital of Zhongshan University from April 1999 to November 2000. All of the samples had been diagnosed as nasopharyngeal poorly differentiated squamous cell carcinoma by the Department of Pathology of the Cancer Hospital. HER-2/neu gene amplification and expression were examined on each sample. Among the 40 patients, 31 were males and 9 were females. The median age of the patients was 45 years old, ranging from 22 to 66 years. According to the (1992) NPC clinical staging criteria, the 40 patients were staged as follow: Ⅰ 2, Ⅱ 16, Ⅲ 11, and Ⅳ 11; T1 7, T2 15, T3 10 and T4 8; N0 10, N1 19, N2 10 and N3 1.

Cell Lines

CNE-2 is a HER-2/neu deficient-expression NPC cell line, which is supplied by the Cancer Research Center of Zhongshan University for Medical Sciences. CNE-2Z is a HER-2/neu overexpressed NPC cell line, which is kindly provided by the Department of Pathology of Guangdong Medical college.

Reagents

Reagents (1)HER-2/neu gene amplification is detected by Vysis Path Vysion™ DNA probe kit, which contains a HER-2/neu probe, an chromosome 17-centromere probe, DAPI, NP-40 and 20×SSC solution. (2)HER-2/neu protein expression is tested by the DAKO Hercep Test™ kit, which includes per-

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oxidase block, rabbit anti-human HER-2/neu antibody (1st Ab), goat anti-rabbit HRP-labelled immunoglobulin (2nd Ab), DAB (substrate chromogen), epitope retrieval solution, wash buffer solution, negative control reagent(containing the same concentration immunoglobulin compared to 1st Ab), and control slides (containing the several positive slides that represent different levels of HER-2 protein expression). (3) RT-PCR: AMV reverse transcriptase (Promega), RNA extraction reagent TRIzol (Gibco-BR) and Taq DNA polymerase (Bocai Company, Shanghai), DNA marker DL-2000 (Bio-biology Ltd. Dalian)

Test Methods

Detection of HER-2/neu gene amplification by FISH with Path Vysion™ DNA probe kit is done in accordance with the manufacturer´s recommended protocol.

Determination of HER-2/neu protein expression by IHC with DAKO Hercep Test™ Kit is carried out according to the manufacturer´s instructions.

RT-PCR: RNA is extracted when the cell reaches the 80% confluence. The ratio of 260/280 of RNA sample is 2.0 when detected by spectrometer. There are two bands of 18s and 28s when running gel. (Fig.1). After DNase treatment, the RNA is amplified by PCR to detect GADPH fragment, which size is 586bp. CNE-2 cell line DNA is used as positive control. The reaction system consists of 2.5 μl 10 × buffer (containing 25 mmol/L $MgCl_2$), 1 μl dNTP (10 mM/each), 0.5 μl Taq DNA polymerase (5 u/μl), 0.5 μl GAPDH primers 1/2 (20 μM/μl), adding water to 25 μl. The PCR program is as follow: initial denature 94℃ 5min, denature 94℃ 30 sec, anneal 55℃ 30 sec, extension 72℃ 30 sec, total 25 cycles, the final extension 72℃ 7min. The PCR products are run on 1.2% agrose gel. After stained with EB, the gel is observed in gel image system. Except for the positive control, there are no 586bp GADPH amplicationn in all RNA samples treated with DNase. The result confirms that there is no DNA contamination in DNase-treated RNA samples (Fig.2). The HER-2/neu gene specific primer pair is designed with PRIMER 3 software according to its mRNA sequence. The forward primer is 5´- GCCCTCATCCACCATAACAC-3´, and the reverse one is 5´-TTCCTCCACGCACTCCTG-3´ (synthesized by Shenggong Ltd, Shanghai). This primer pair will amplify the 234bp cDNA fragment located at 1569 to 1808nt. Draw 8 μg total RNA, add 10 μl 5×RT buffer, 1 μl dNTP, 6 μl AMV (10u/μl), 3 μl Oligo d(T) primer (0.5 μg/μl), 2.0 μl random primer (0.5 μg/μl), finally add water to 40 μl, mix, incubate 1 hour at 42℃, store at −20℃. Draw 1 μl above reverse transcription reaction product as template, add 2.5 μl 10 × buffer, 1 μl dNTP, 0.5 μl Taq DNA polymerase (5 u/μl), 1μl HER-2/neu-specific primers (20 μM/μl), finally add water to 24 μl. The PCR program is as follow: initial denature 94℃ 5min, denature 94℃ 30sec, anneal 55℃ 30sec, extension 72℃ 30sec, total 28 cycles, the final extension 72℃ 7min. After 5 cycles, 0.5 μl GAPDH primers 1and 2 are added. The amplified products are separated on 1.2% agrose gel. After stained with EB, the gel is observed in gel image system.

Results Determination Standards

HER-2/neu gene amplification: The FISH kit contains two labeled DNA probe. The locus-specific HER-2/neu probe is labeled in spectrumOrange. The chromosome 17-centromere probe is labeled in spectrum green. The results are expressed as the average ratio of the HER 2/chromosome 17 signals. Ratio of greater than or equal to 2.0 indicates HER 2 gene amplification. Ration of lower than 2.0 demonstrates no HER gene amplification.

HER-2/neu gene expression: Calculate the percentage of positive cells, which have membrane staining, of total 500 counted tumor cells under microscope. The positive cells are graded by the membrane staining intensity and percentage. The HER-2/neu protein overexpression is interpreted as a moderate to strong complete membrane staining in more than 10% of tumor cells.

RT-PCR detection: With the GAPDH products used as internal control, the brightness of HER-2/neu gene products is compared among different samples. The more the brightness, the more the amplified products.

Statistical Analysis

SPSS 8.0 statistical software is used for statistical analysis. The correlation between the HER-2/neu gene amplification or expression and the original, lymph node metastasis and clinical NPC stages is analyzed by the Chi-square test.

RESULTS

Amplication of HER-2/neu gene in NPC tis-

sues Though no amplication of HER-2/neu gene was detected in all 40 NPC samples (Fig.1 –3), change of chromosome structure and number was found in tumor cells, among which there are 3 sporadic loss of chromosome and 4 sporadic multiplicate chromosome.

Expression of HER-2/neu gene in NPC tissues Overexpression of HER-2/neu gene was detected in 18 of the 40 NPC samples with an overexpression rate of 45%(Fig.4), and there is no expression of HER-2/neu gene in the other 22 NPC samples.

The relationship between expression of HER-2/neu gene and primary disease staging, lymph node metastasis staging and clinical staging: After analyzing the relationship between expression of HER-2/neu gene and primary disease staging, lymph node metastasis staging and clinical staging (Table 1), no correlation was found between each other.

Amplication of HER–2/neu gene in NPC cell lines No singal of amplication of HER-2/neu gene was detected in CNE-2 and CNE-2Z (Fig.5), and therefore these two NPC cell lines did not have amplication of HER-2/neu gene.

Expression of HER-2/neu gene in NPC cell lines Membrane staining was positive in 90% of cell line CNE-2Z using IHC (Fig.6), and thus this indicated overexpression of HER/neu gene in it. While in cell line CNE-2, only sporadic membrane staining was positive in 10% cells, and this meant no overexpression existed in it. Amplication of GAPDH was detected in both CNE-2Z and CNE-2 using RT-PCR. While many amplified HER/neu gene was detected in CNE-2Z, only few-2/neu mRNA was higher in CNE-2Z than in CNE-2 (Fig.7).

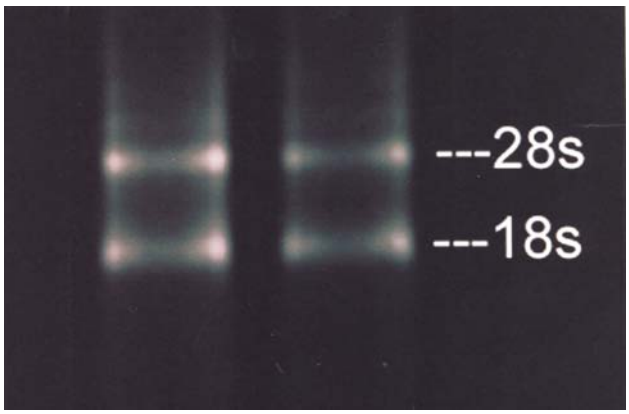


Fig.1 Electrophoregram of overall RNA treated by DNase

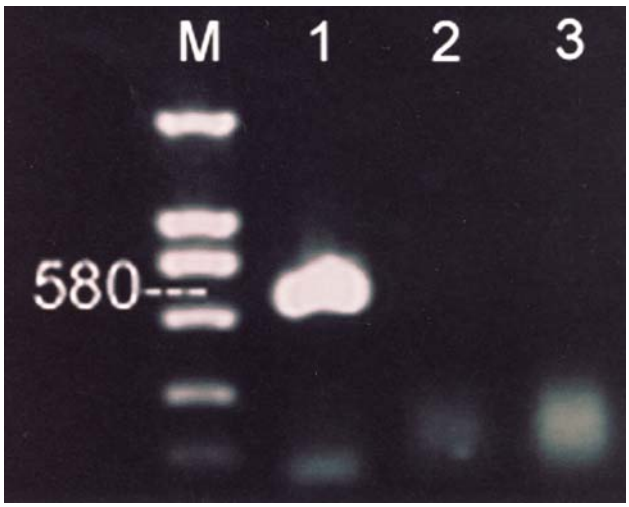


Fig. 2 PCR reaction of overall RNA GAPDH treated by DNase

1 mean control DNA, 580bp fragment amplificated,

2–3 mean overall RNA, no product amplificated means no DNA contamination in RNA.

Table 1 HER–2/neu gene expression and clinical stage and primary tumor and neck lymph nodes stage

	Patients number (40)	HER–2/neu expression		P
		overexpression	deficient expression	
Clinical stage				
I/II	18	8	10	
III/IV	22	11	12	0.95
Primany tumor				
T1	7	1	6	
T2	15	7	8	0.15
T3	10	5	5	
T4	8	6	2	
Neck lymph nodes				
N0	10	5	5	
N1	19	9	10	
N2	10	5	5	1.00
N3	1	0	1	

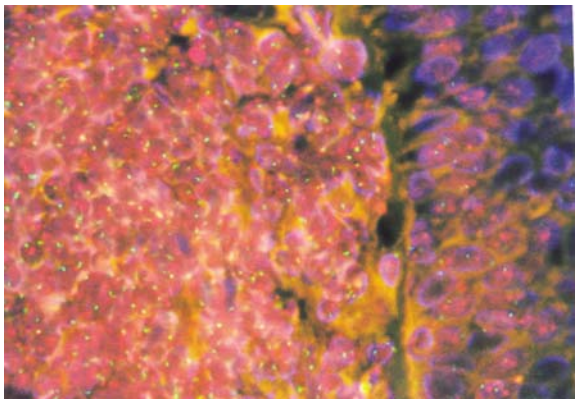
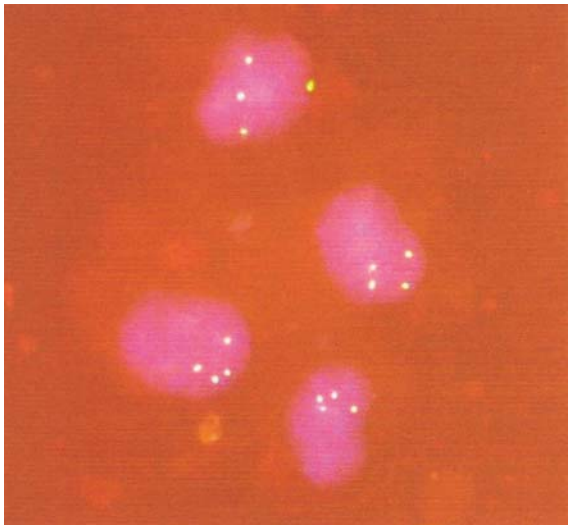
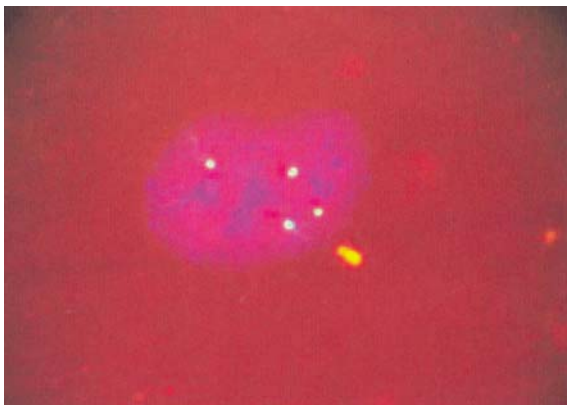


Fig.3 The result of HER-2/neu gene amplification tested in NPC tissues

Spectrumorange and spectrumgreen signals display in tumor cells, signal ration of lower than 2.0 in the same cell demonstrates no HER-2/neu gene amplification (DAPIx100).



A



B

Fig.5 The results of HER-2/neu gene amplification tested by FISH in NPC cell lines

A is CNE-2Z cell line and B is CNE-2 cell line, SpectrumOrange and spectrumgreen hybrid signals display in cells, identical signal numbers as 4 mean no HER-2/neu gene amplification.

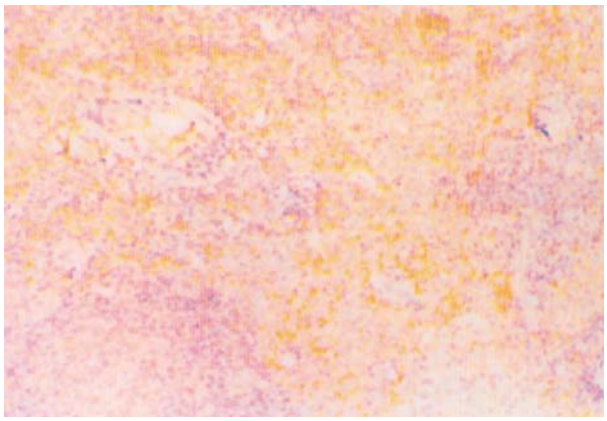
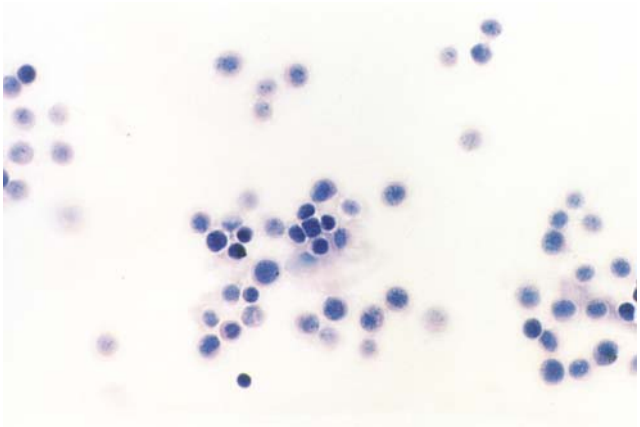
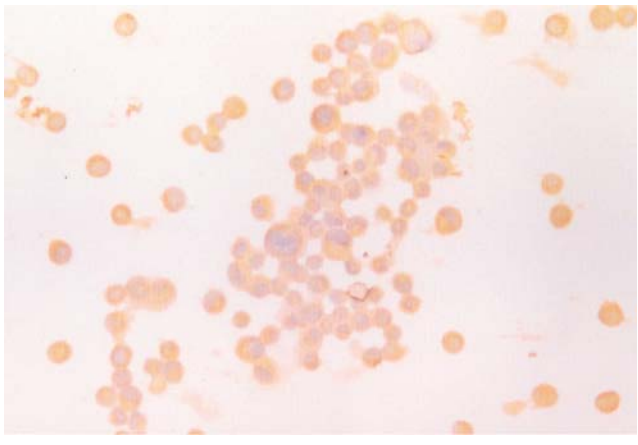


Fig. 4 The result of HER-2/neu gene expression tested by IHC in NPC tissues

Brown-yellow staining is mostly located to tumor cells membrane, it means HER-2/neu gene strong Expression (HEx40).



A



B

Fig.6 The results of HER-2/neu gene expression tested by IHC in NPC cell lines

A is HER-2/neu gene positive expression of CNE-2Z cell line and B is HER-2/neu gene negative expression in CNE-2 cell line.

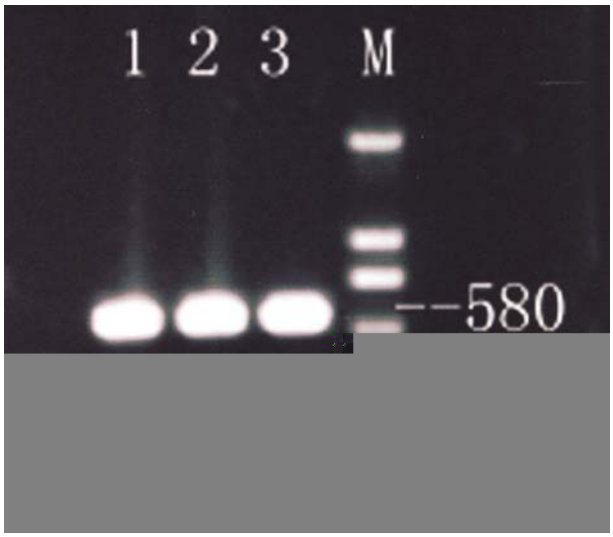


Fig.7 Electrophoregram of HER-2/neu gene semi-quantity RT-PCR amplificated in NPC cell lines
GAPDH products used as internal control, 580 site is GAPDH, 234 site is HER-2/neu amplification product, 1 is CNE-2Z cell line and 2 is CN-2 cell line, brightness in 234 site means HER-2/neu gene expression level, The more the brightness, the more the expression. The fig display difference of brightness in 234 site in 1 and 2.

DISSCUSION

No amplication of HER-2/nue in NPC was detected in our research. Up to now, only few research about HER-2/neu amplication were performed, among which reported amplication of HER-2/neu in NPC using PCR[2], and the result was contrary to ours. Several methods were used in examing gene amplication, including Southern Blotting, PCR, fluorecent hybride in situ which is most widely used in clinical research. fluorecent hybride in situ is comparatively simple because of available commercialized examination reagent, and its results were accurate and quantitative; While PCR is complicated because of no available commercialized examination reagent, and its results which were affected by many factors and only half quantitative were not so accurate as the former ones. Our research adopted standard fluorecent hybride in situ reagent for HER-2/neu gene amplication recommended by FDA, so our results were reliable.

In evaluating the prognoses of tumor, amplication and overexpression of HER-2/neu gene in terms of examination and clinical research in breast cancer are valuable and highly in accordance with each other [3-6], and amplication is more instructive because of its biological meaning and its more accurate results. Gene amplication which often occurs in late stage tumor facilitates tumor growth. Accuracy of gene amplication and expression which use FISH and IHC respectively had some difference because of different examination target, different examination principle and restriction of methods evaluation. Results of gene amplicaton using FISH were more accurate. Expression of HER-2/neu was used to predict prognoses in NPC because no amplication was detected in NPC, and our result indicated there were no correlation between expression of HER-2/neu and primary disease staging, lymph node metastasis staging and clinical staging. The same was true of the relationship between expression of HER-2/neu and five year overall survival rate, three year disease free survival rate in our another research. According to our research above, we considered there was no amplication of HER-2/neu gene in NPC and no relationship existed between expression of HER-2/neu and several important prognostic indicator, and therefore HER-2/neu gene may not be a valuable prognostic indicator in NPC.

The relationship between amplication and overexpression of oncogene is often linear, that is gene amplication can cause corresponding gene overexpression which is induced by mRNA expression affected by the gene change during transcription which involves transcription regulation through three steps including pre-, during and post transcription regulation and change in each step can cause change in mRNA. According to our results, mRNA over expression existed in CNE-2Z which has overexpression of HER-2/neu gene, and this indicated overexpression of HER-2/neu gene in NPC was related to mRNA overexpression. A research which examed expression of mRNA and protein of HER-2/neu gene in NPC tissue with expression rate of 84% in both using IHC and hybride in situ also considered over expression of HER-2/neu gene protein was caused by that of HER-2/neu gene mRNA and this result was consistent with ours [7]. So we concluded that the occurrence of HER-2/neu gene over expression was related to mis-regulation of gene transcription.

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