Ile/Ile Homozygosity at Codon 655 of HER2 in Schwannoma

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Abstract-

Purpose: This study aimed to determine the genetic role of HER2, one of the epidermal growth factor receptor (EGFR) family, in schwannoma. The latter is a neogrowth of myelin-producing Schwann cells in peripheral nerves, inducible by N-nitrosoethylurea in animals with mutation in the neu gene (homologous gene of human HER2 protein).

Methods: In this study we obtained genomic DNA samples from tissue blocks of schwannoma, initially by xylene treatment and alcohol extraction, followed by use of the DNA extraction kit. Evaluation of this genetic polymorphism in our subjects was conducted by direct nucleotide sequencing or restriction enzyme analyses after PCR work.

Results: There were thirty extracted DNA samples from tissue blocks of schwannoma, and all were Ile/Ile homozygotes after genotype analyses. Two individuals received the leukocyte DNA extraction after peripheral blood sampling, both showing Ile/Ile homozygosity. This study gave the impression of an association of the HER2 polymorphism at codon 655 with tumorigenesis of schwannoma. Although the majority of the Taiwanese showed Ile/Ile homozygosity (about 83%), the present study revealed a 100% carriage rate among the tissue blocks from our subjects with schwannoma.

Conclusion: Ile/Ile homozygosity at codon 655 of HER2 in schwannoma may imply some role in tumorigenesis of Ile655Val allele of HER2 in this nerve tumor.

Key Words: schwannoma, HER2, codon 655 polymorphism, Ile/Ile homozygosity

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INTRODUCTION

Schwannoma ranks the third in the primary nerve cell neoplasms, arising from the Schwann cells in the peripheral nerve system. Most of them are unilateral; but bilateral vestibular schwannoma and multiple spinal nerve root involvement may occur in subjects with NF2 mutation[1]. Surgical removal is the first choice of treat-

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ment, including the use of gamma knife radiosurgery[2]. Morphology of Schwann cells is related to the binding of schwannomin (also known as merlin), the gene product of NF2, with the adaptor paxillin on the cell membranes[3]. There were evidences of overexpression or activation of growth factor receptors, including those for the vascular endothelial and platelet-derived growth factors[4,5]. In addition, neuregulin (NRG) and erbB2/erbB3 expression were also noted[6-8], with the possibility of an existing an autocrine loop[7].

HER2, also known as erbB2 or HER2/neu, has a molecular weight of 185 KDa, and it belongs to the family of epidermal growth factor receptors (EGFR). HER2 is associated with tumorigenesis, metastasis and clinical prognosis of cancers. Cell signaling via dimerization on ligand binding, followed by auto-phosphorylation by the tyrosine kinase domain of HER2 activates the downstream target proteins, such as mTOR, Src, STAT, MAPK[9]. Oncogenic murine HER2/neu was initially found by transplacental injection of nitrosoethylurea (NEU)[10,11], and the single point mutation from valine to glutamic acid at codon 664 of the transmembrane domain of rat HER2 resulted in elevated tyrosine kinase activity and cell transformation[12]. The same gene mutation was seen in the spontaneous peripheral nerve sheath tumor in animals[13]. This hotspot in the transmembrane domain is corresponded to the codon 655 in human, and the polymorphic valine (Val) allele was found to increase the risk for breast cancer, especially in individuals younger than 45 years of age [14,15].

Recently the role of erbB receptors has caught the attention on the nerve development [16,17]. In the peripheral nerve system, the HER2 (erbB2) and erbB3 guided the expansion of Schwann cells on neural crest growth and myelination [18], with up-regulation and activation in Schwann cells on sciatic nerve injury [19,20], and also a role in the formation of neuromuscular junction [21,22]. During the development of the central nerve system, HER2 induced the radial glia transformation [23] and the dendritic spine maturation [24,25]. With the above findings, recent evidence of erbB2-dependent glial cell growth by the schwannomin [26] and constitutive expression of phosphorylated HER2 in schwannoma [8] gave hints of the role of erbB2 in the formation of schwannoma.

Tumorigenesis of schwannoma may have genetic vulnerability related to codon 655 of exon 17 in HER2 (Ile655Val). In this study, we investigated the polymorphic allelic distribution at codon 655 of HER2 among schwannoma samples from our subjects. The results encouraged us to explore the role of HER2 in formation of benign tumors.

METHODS

Samples and Patients

Schwannoma was diagnosed independently by the two pathologists (Suen JH and Li JW), according to the microscopic finding of spindle cell proliferation of Antoni A or B patterns. Medical records were reviewed and these patients had one tumor on physical examinations, instead of the swannomatosis or neurofibromatosis. We got the informed consents, brief personal and family histories. In addition, the blood leukocytes in the puffy coat were stocked after venopuncture from the subjects without schwannoma (125 samples) as non-disease controls. These controls, also with consents, included those with strokes, diabetes mellitus, hypertension, gout, osteoarthritis and spine disorders.

DNA Extraction

Tissue blocks were subjected to xylene to dissolve the paraffin, followed by treatment of 96-100% ethanol. DNA extraction was conducted with QIAamp DNA FFPE Tissue Kit (QIAGEN), according to the manufacture's instructions. The leukocyte DNA was extracted from the peripheral blood by QIAamp DNA Blood Midi kit (QIAGEN) from the peripheral blood samples from the two subjects with schwannoma and controls.

Genotyping of Codon 655 of HER2

All the DNA samples from tissues and leukocytes of the two individuals with schwanoma and controls were subjected to polymerase chain reaction (PCR). PCR was performed using a template DNA (20-100 ng) and specific primer pair for codon 655 (NCBI access number: NM 004448). The primer pair was designed as HN-5: 5'-

Table. The genotype distribution at codon 655 of HER2 among the controls. As comparison with the published reports, there was low prevalence of Val carriers among Taiwanese and the Eastern Asians

Population	Numbers of subjects	HER2 Ile655Val polymorphism, % (*n*)		Reference
		Ile/Ile	Val carriers (Ile/Val + Val/Val)	
Taiwan	125	84.8 (106)	15.2 (19)	This study
Taiwan	318	85.8 (273)	14.2 (45)	Lee, *et al*. 2008
Shanhai	359	78.0 (280)	22.0 (79)	Xie, *et al*. 2000
Korea	979	74.3 (727)	25.7 (252)	Han, *et al*. 2005
Japan	184	73.9 (136)	26.1 (48)	Hishida, *et al*. 2002
USA	1667	58.8 (980)	41.2 (687)	Cox, *et al*. 2005
Gemany	960	54.7 (525)	45.3 (435)	Frank, *et al*. 2005

Ile, isoleucine; Val, valine

AGA GAG CCA GCC CTC TGA CGT CCA T-3'; and HN-4: 5'-TCC GTT TCC TGC AGC AGT CTC CGC A-3' (PCR product with 148 bp)[27]. The PCR volume of 70 µl included *Taq* polymerase (1.0 unit), primers (20 pmol/µl of each primer pairs), each dNTP (200 µM), and 10X buffer (7 µl) with $MgCl_2$ (3.0 mM) in working solution. On the thermocycler (GeneAmp PCR system 9700, Applied Biosystems), the temperature schedule was as follows: 94 ˚C for 1 min, 45 cycles of 94 ˚C for 30 seconds, 65 ˚C for 30 seconds, and 72 ˚C for 20 seconds, with final extension by 72 ˚C for 10 min and then soaking at 4 ˚C. Restriction enzyme digestion with *Bsm*AI (New England BioLabs) was performed at 55 ˚C for 4 hours to identify the Val allele after complete digestion. To confirm the heterozygosity for samples with partial digestion, the PCR products were subjective to sequencing by HN-5 and HN-6, 5'-TTC CGG ATC TTC TGC TGC CGT CGC T-3'.

Statistical Analysis

Chi-square analysis and Hardy-Weinberg equilibrium were applied to check the allelic polymorphic distribution and compile statistics.

RESULTS

We reviewed 33 tissue slides of schwannoma from 2002 to 2007, and only thirty with DNA extraction were feasible for PCR work. There were no family histories of the peripheral nerve tumors among these subjects, and they did not find other schwannoma mass or tumor recurrence on self examinations. These 30 samples came from 11 females and 19 males, mainly excised from the peripheral nerves, 12 from axial regions (including the trunk, chest, neck and scalp), 16 from the limbs and 2 from the intracranial cerebellopontine angle regions. The ages of these patients on tumor resections ranged from 18 to 70 years, with mean age of 41.2 years. On our telephone surveys during the following 3 years, none had *de novo* CNS tumors.

Twenty-one of the 30 PCR products (70.0%) were subjected to nucleotide sequencing, with reading as homozygosity of Ile/Ile. The rest of the products also revealed homozygosity of Ile/Ile by the restriction fragment length polymorphism (RFLP). DNA samples from peripheral leukocytes were obtained from two subjects with previous schwannomas, one 54-year-old lady and the other 45-year-old man. Both two blood DNA samples exhibited Ile/Ile homozygosity at codon 655 of HER2 gene, as the genotype from their tissue blocks. Besides, leukocyte DNA from 125 control subjects were also collected, and the analytic results disclosed 106 with Ile/Ile homozygosity (84.8%), 17 with Ile/Val heterozygosity (13.6%), and only 2 Val/Val homozygotes (1.6%) (15.2% as the Val carrier rate, Table). With the unique genotype in our samples, there was significant difference (χ^2 =5.197, p = 0.02, df = 1) between the schwannoma group and the controls.

DISCUSSION

HER2 contributes to the cancer risk with increased gene copies on genomic analyses [28]. In addition, there was evidence of polymorphism of HER2 associated with cancers, especially breast cancer with a higher frequency correlation to the Val-allele carriers at codon 655 of HER2 gene. The phenomena had no racial differences [14,29-33]. From two prior reports in Taiwan and China, the ethnic Hans showed the Ile/Ile homozygosity as the majority (controls, 85.8% and 78.0% respectively, Table) [14,29]. The former about Taiwanese is similar to our findings among the controls (84.8%). However, all subjects with benign schwannoma in this study were Ile/Ile homozygotes. There is increased significance in this unique genetic distribution, and the possible alternative role of HER2 in benign peripheral nerve tumors. Although both of Val and Ile have non-polar side chains, polymorphism at the hotspot codon 655 has its biological significance in forming a stable conformation and kinase activity on dimerization [34], possibly related to the length of alkyl chains.

Schwannoma may occur independently or in association with neurofibromatosis type 2 (NF2), the latter with a disease prevalence of about 1:60,000 [35]. NF2 mutations may cause vestibular or other peripheral schwannoma, menigioma, glioma and neurofibroma. In addition, mesothelioma [36] and renal cell carcinoma [37] were also known to be related to NF2 mutations. In this study all subjects did not have *de novo* CNS tumors. We did not survey the genomic status of the NF2 gene, for the sake of clinical phenotype of tumors. In Drosophila, NF2 may control the abundance of membrane receptor proteins, including EGFR [38]. Recently, the tumor suppressor NF2 has been reported to exhibit the contact inhibition by EGFR internalization and signaling [39,40]. Thus the interrelationship between NF2 and HER2 may co-regulate the proliferation and balance of the steady function of Schwann cells. Immortalized Schwann cell line with different allele expressions may be a model to approach this issue.

Polymorphic association of one gene with an illness may suggest the genetic contribution to pathogenesis. Although the evidence is not as strong as the mutations with gain or loss of functions, this study still has clinical significance. We found a unique distribution of HER2 codon 655 among our samples. It is different from the prior findings of higher association of Val allele with breast cancer among Hans (Lee's study, χ^2 =7.186, p = 0.072, df = 1; Xie's study, χ^2 =11.483, p = 0.001, df = 1) [14,29]. Some supposition may rise, such as that Val allele may have higher activity on cell survival and/or anti-apoptosis in malignant cells. However, in benign schwannoma we wonder that Val allele acts in a protective role against the formation of schwannoma. The genotype of Ile/Ile may be regarded as the wild type in the general population and Val allele appears while the evolution with the tumor events occurs. Our control group exhibited the low prevalence of Val carriers, however of the higher one among the Caucasians (Table). From the viewpoint of genetic contribution, it implies that the tumors with homozygosity of Ile/Ile run the benign course.

Some limitations exist in our present hospital-based study. First, the recruitment difficulty of the schwannoma samples resulted in limited sample size and the possibility of sampling bias. Results from the thirty samples still showed a relevance of Ile/Ile homozygosity at codon 655 of HER2. Second, we did not stain HER2 (or erbB2) in the schwannoma to check for expression levels of HER2 on tumor bulk. High expression of HER2 including increased gene copies may imply the malignant tendency. However, the benign clinical feature of schwannoma may exclude the possibility of over-expression of HER2 in schwannoma. Third, we did not compare genotypes of schwannoma with tumors of other etiologies for disease controls, for example, traumatic neuroma, malignant peripheral nerve sheath tumors or other non-neural tumors. The work became more challenging to obtain the non-blood tissues from our controls. In addition, the scanty normal tissues near the tumor bulks yielded little DNA to conduct further PCR works. However, two leukocytic DNA samples from our patients received the genotypic analyses, which did not differ from the findings of schwannoma samples. It requires further investigations to explore the possible difference and signifi-

cance of HER2 in the pathogenesis of schwannoma. Last, our study did not investigate the NF2 gene. HER2 and NF2 may act reciprocally as drive-brake roles in cells, but gain-of-function effect occurs in cancers carrying mutations in these two genes. The cross talk of these genes may involve other molecules as the partners or mediators. Overall, this study investigated HER2 with a unique genotype at codon 655 among our samples and gave the modest possibility for us to explain the role of HER2 in schwannoma. Further works may be required to investigate the interrelationship between this peripheral nerve tumor and oncogenes, possibly via approaches by genomic and proteomic expression profilings.

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