Overexpression of ERBB-2 was more frequently detected in malignant than benign pheochromocytomas by multiplex ligation-dependent probe amplification and immunohistochemistry

WenQi Yuan[1,2], WeiQinq Wang[1,3], Bin Cui[1,2], TingWei Su[1], Yan Ge[1,2], Lei Jiang[1], WeiWei Zhou[1] and Guang Ning[1,2,3]

[1]Department of Endocrine and Metabolic Diseases, School of Medicine, Shanghai Clinical Center for Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai JiaoTong University, 197 Ruijin Er Lu, Shanghai 200025, People's Republic of China
[2]Laboratory of Endocrine and Metabolic Diseases, Shanghai JiaoTong University School of Medicine and Shanghai Institutes for Biological Sciences, Institute of Health Sciences, Chinese Academy of Sciences, Shanghai 200025, China
[3]Division of Endocrine and Metabolic Diseases, Shanghai JiaoTong University School of Medicine, E-Institute of Shanghai Universities, Ruijin Hospital, Shanghai 200025, China

(Correspondence should be addressed to G Ning; Email: guangning@medmail.com.cn)

W Yuan, W Wang and B Cui contributed equally to this work

Abstract

To analyze the genetic alterations of pheochromocytomas and evaluate the difference among malignant, extra-adrenal, and benign pheochromocytomas. Forty-three tumor samples were tested for genetic changes using multiplex ligation-dependent probe amplification. Among them, 39 samples were available for protein expression analysis by immunohistochemistry (IHC). All 43 patients (24 women and 19 men; mean age 44.6 ± 13.6 years; range 18–75 years; 9 with malignant, 7 extra-adrenal, and 27 benign) showed multiple copy number losses or gains. The average copy number change was 13.10 in malignant, 13.93 in benign, and 13.47 in paraganglioma patients. There is no significant difference among the three groups of pheochromocytomas. However, we discovered that in the malignant pheochromocytomas, 6 of the 9 patients (67%) showed erythroblastic leukemia viral oncogene homolog 2 (*ERBB-2*) oncogene gain, whereas only 12 of the 34 (35%) identified change in the benign and extra-adrenal pheochromocytomas. Further, IHC confirmed that ERBB-2-positive staining was more frequent and stronger in malignant pheochromocytomas than in benign and extra-adrenal pheochromocytomas. Our study illustrates the chromosomal changes of the whole genome of Chinese pheochromocytoma patients. The results suggest that there may be certain progression of genetic events that involves chromosomes 1p, 3p, 6p, 11q, 12q, 17q, and 19q in the development of pheochromocytomas, and the activation of ERBB-2 located on chromosome 17q is an important and early event in the malignancy development of these tumor types. The overexpression of ERBB-2 identified by IHC suggested that this oncogene could be associated with the malignancy of pheochromocytomas and paragangliomas.

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Introduction

Pheochromocytomas are rare neoplasms of the chromaffin cells that derived from the embryonic neural crest. They usually occur within the adrenal medulla; however, extra-adrenal pheochromocytomas may arise in ganglia of the sympathetic nervous system. Both the clinical and biochemical features of

pheochromocytomas are mostly the result of the overproduction of catecholamines. In sporadic pheochromocytomas and paragangliomas, ∼20% of them are associated with germline mutations that lead to paraganglioma syndrome (succinate dehydrogenase subunit B/C/D; SDHB, SDHC, and SDHD), von Hippel–Lindau syndrome (VHL), and multiple endocrine neoplasia type II (MEN2) and, to a much lesser extent, type 1 neurofibromatosis (NF1; Casanova *et al*. 1993, Maher & Kaelin 1997, Neumann *et al*. 2002, Bausch *et al*. 2006). Studies of genomic alterations in primary pheochromocytomas using comparative genomic hybridization (CGH) have shown the loss of chromosomes 1p, 3p, 3q, 11p, 17p, and 22q at various frequencies, suggesting the existence of tumor suppressor gene loci in these locations (Yokogoshi *et al*. 1990, Moley *et al*. 1992, Shin *et al*. 1993, Zeiger *et al*. 1995, Edstrom *et al*. 2000). Gain of chromosomes 17q and 11q13 was more frequently identified in malignant tumors (August *et al*. 2004, Eisenhofer *et al*. 2004, Cascón *et al*. 2005). However, it is difficult to identify which specific gene was involved in the pathogenesis of pheochromocytoma by CGH analysis. Until recently, there was lack of knowledge about the exact genes involved in the pathogenesis of sporadic pheochromocytoma. To find new candidate genes, we performed multiplex ligation-dependent probe amplification (MLPA) analysis in pheochromocytomas. MLPA is a new technique used to measure the copy number of up to 45 nucleic acid sequences in one single reaction (Schouten *et al*. 2002). This method relies on sequence-specific probe hybridization to genomic DNA followed by multiplex-PCR amplification of the hybridized probe and semiquantitative analysis of the resulting PCR products. This method is easy to perform and could offer an alternative to CGH studies (Hogervorst *et al*. 2003, Bremmer *et al*. 2005, van Dijk *et al*. 2005, Postma *et al*. 2005).

We investigated 43 pheochromocytoma tissue samples using MLPA analysis and found that several genes may contribute to the tumorigenesis of pheochromocytoma or malignancy of the disease. These results were similar to those detected by CGH analysis. The most obvious was the gain of erythroblastic leukemia viral oncogene homolog 2 (*ERBB-2*) oncogene located on chromosome 17q that was more common in malignant pheochromocytomas. Moreover, we analyzed the *ERBB-2* oncogene products from pheochromocytomas by immunohistochemistry (IHC); the results confirmed the previous genetic analysis.

Materials and methods

Patients and samples

Forty-three patients (24 women and 19 men; mean age 44.6 ± 13.6 years; range 18–75 years) operatively proven with sporadic pheochromocytomas were included in this study. The diagnosis of pheochromocytoma was confirmed on the basis of clinical manifestations including persistent or paroxysmal hypertension with the classic triad of severe headaches, palpitations, and diaphoresis, biochemical features including serum and urinary metanephrine (MN), nor-MN (NMN), and positive CT or ^{131}I-MIBG scan. The diagnosis of malignant pheochromocytoma was based on the presence of distant metastases or lymph node infiltrations, that is, the presence of chromaffin tissue at sites where this tissue is normally absent (Eisenhofer *et al*. 1999, Weise *et al*. 2002, Mittendorf *et al*. 2007). Of the 43 patients, 9 had malignant disease, 7 were paragangliomas of the abdomen, and the rest of the 27 patients were with benign pheochromocytomas. All of the patients were operated in between 2002 and 2006 and clinically monitored at the Shanghai Clinical Center for Endocrine and Metabolic Diseases in the Ruijin Hospital. The study was approved by the Ethics Committee of the Ruijin Hospital, Shanghai JiaoTong University School of Medicine.

Tumor tissues were snap-frozen in liquid nitrogen immediately after surgery. Genomic DNA was extracted from fresh-frozen tumor specimens by standard procedures using a Qiagen DNA extraction Kit (Qiagen) according to manufacturer's guide. All pheochromocytomas were histologically analyzed before DNA extraction to ensure preparation of a population of the tumor cells without cross-contamination with the normal cells.

Multiplex ligation-dependent probe amplification

We performed MLPA analysis using SALSA P005 and P006 Chromosomal Aberration MLPA Kits (MRC-Holland, Amsterdam, The Netherlands; details available at http://www.mlpa.com) and following the manufacturer's protocol. The P005 and P006 kits include 76 genes loci, which were selected because of their reported involvement in cancer and can span all 23 chromosomes including the X and the Y. With the MLPA, DNA samples were heated at 98 °C for 5 min; after the addition of the probe mix, samples were heated for 1 min at 95 °C and then incubated for 16 h at 60 °C; ligation of the annealed oligonucleotide probes was performed for 15 min at 54 °C with Ligase-65

enzyme. Multiplex PCR amplification was carried out using Cy5-labeled primers, dNTPs, and SALSA polymerase. PCR was performed for 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C. All the reactions were carried out in PTC-225 DNA Engine Tetrad (MJ Research Inc., San Francisco, CA, USA). PCR products were analyzed using Beckman Coulter CEQ 8800 sequencer (Beckman Coulter, Fullerton, CA, USA). Data analysis was performed with fragment analysis module. Normal control DNA of males and females was also used in the same reaction.

MLPA data analysis

The Gene Scan data of sizes and peak height of multiplex PCR products were exported to an Excel file. All the expected MLPA products were normalized by dividing each peak height by the combined peak height of all peaks in that lane (relative peak height). The relative copy number for each probe was expressed as a ratio of the relative peak height for each locus of the sample to that of the normal sex-matched control. The reference median peak heights were obtained from normal tissue samples, each of which was analyzed at least thrice independently. Male and female samples were compared with male and female control samples respectively for quantitation of X- and Y-linked probes. The ratio <0.7 and >1.35 was considered as a loss and a gain respectively.

Immunohistochemistry

IHC analysis was carried out to verify the gene changes detected by MLPA. To minimize sampling variability, the following steps were taken. All samples (control and experimental) were 1) run in parallel and each treated identically, 2) examinedunder identical conditions, and 3) repeated at least twice. Five micrometer sections were cut and deparaffinized. Then the sections were immersed in sodium citrate buffer adjusted to pH 6.0 and heated in a microwave oven for 5 min at 100 °C, next treated with 0.3% H_2O_2 for 30 min to inhibit peroxidases, then blocked with 2% normal rabbit serum for 30 min. The slides were incubated overnight in a solution of ERBB-2 monoclonal antibody (DAKO Rabbit anti-human ERBB-2 oncoprotein) diluted to 1:200 with the same buffer. The primary antibody binding was demonstrated with standard avidin–biotin-peroxidase complex technique (vector pk-7200). The slides were developed using diaminobenzidine and counterstained with hematoxylin. Breast carcinoma tissue, known to express the ERBB-2 protein, was used as the positive control. Negative controls were incubated with no primary antibody. The results were evaluated quantitatively and divided into four groups (0, negative; $+$, $<30\%$ positive rate; $++$, 30–60% positive rate; and $+++$, $>60\%$ positive rate). The data were entered into a ZEISS Axioplan 2 microcomputer, and statistical analysis was carried out using KS400 3.0 statistical package. The Kruskal–Wallis test was used to evaluate the relationship among the percentage of ERBB-2 immunoreactivity (all four groups) in the benign, extra-adrenal, and malignant pheochromocytomas.

Results

Clinical characteristics

Forty-three patients were pathologically diagnosed with pheochromocytoma or abdominal paraganglioma. The median age at diagnosis was 44.6 ± 13.6 years (range 18–75 years) with a male to female sex distribution of 56 and 44%. Tumors in 16 patients were on the left adrenal, 17 on the right, 1 with bilateral tumor, and another 9 were with paragangliomas. Of the 43 patients, 18 (42%) showed one of the classical triad, 11 (25%) showed two, and 7 (16%) showed all the three triads. However, there were seven patients with no typical symptoms of pheochromocytoma. Elevated serum MN and/or NMN were seen in 95% of the patients. Malignant tumor was seen in 21% of the patients (Table 1). In 34 benign samples, 12 showed gain of *ERBB-2* with the average tumor size of 77.1 mm, while the rest 22 showed normal *ERBB-2* gene with the average tumor size of 49.1 mm ($T=2.729$; $P=0.01$). The clinical characteristics of those malignant tumors were shown in Table 2. All the analyzed tumor tissues were derived from the primary lesions.

Table 1 The clinical features of three different types of pheochromocytomas

	Malignant	Adrenal benign	Paraganglioma benign
Number of patients	9	27	7
Sex (M/F)	2/5	13/14	2/5
Age (average $\pm$ std)	41.1 $\pm$ 6.4	45.7 $\pm$ 15.7	44.8 $\pm$ 13.9
Tumor size (mm)	52.6 $\pm$ 19.7	66.3 $\pm$ 33.1	40.9 $\pm$ 15.2

Table 2 The clinical characteristics of malignant patients

Case	Sex (M/F)	Age (years)	Operation times	MN/NMN (pg/ml)	Tumor size (mm)	Metastatic location
5	F	43	3	353.4/30.9	48×40×43	LN
48	F	43	2	167.7/1414.9	40×30×30	LN
68	F	40	2	202.5/585.9	60×50×30	Liver
75	M	40	1	62.6/4669.4	60×50×40	Lung
49	F	44	2	47.2/2172.3	30×10×10	Kidney
67	F	52	2	42.5/5371.6	85×60×30	Omentum
25	F	38	2	8861.3/104.6	75×32×30	Brain
6	M	31	2	170.8/3942.2	50×45×40	LN
72	M	39	1	NA	25×10×10	LN

NA, not available; LN, lymph node. Reference range: NM, 14.0–90.0 pg/ml and NMN, 19–121 pg/ml.

MLPA

Genome-wide analysis suggested that all 43 pheochromocytoma specimens, including both malignant and benign ones, showed multiple copy number losses or gains. In the malignant, benign pheochromocytomas, and in paragangliomas, the total number of aberrations in each tumor ranged from 6 to 29, 3 to 31, and 7 to 21 respectively, and the average copy number changes were 13.10, 13.93, and 13.47 respectively. Hence, there is no significant difference among the three groups (Fig. 1).
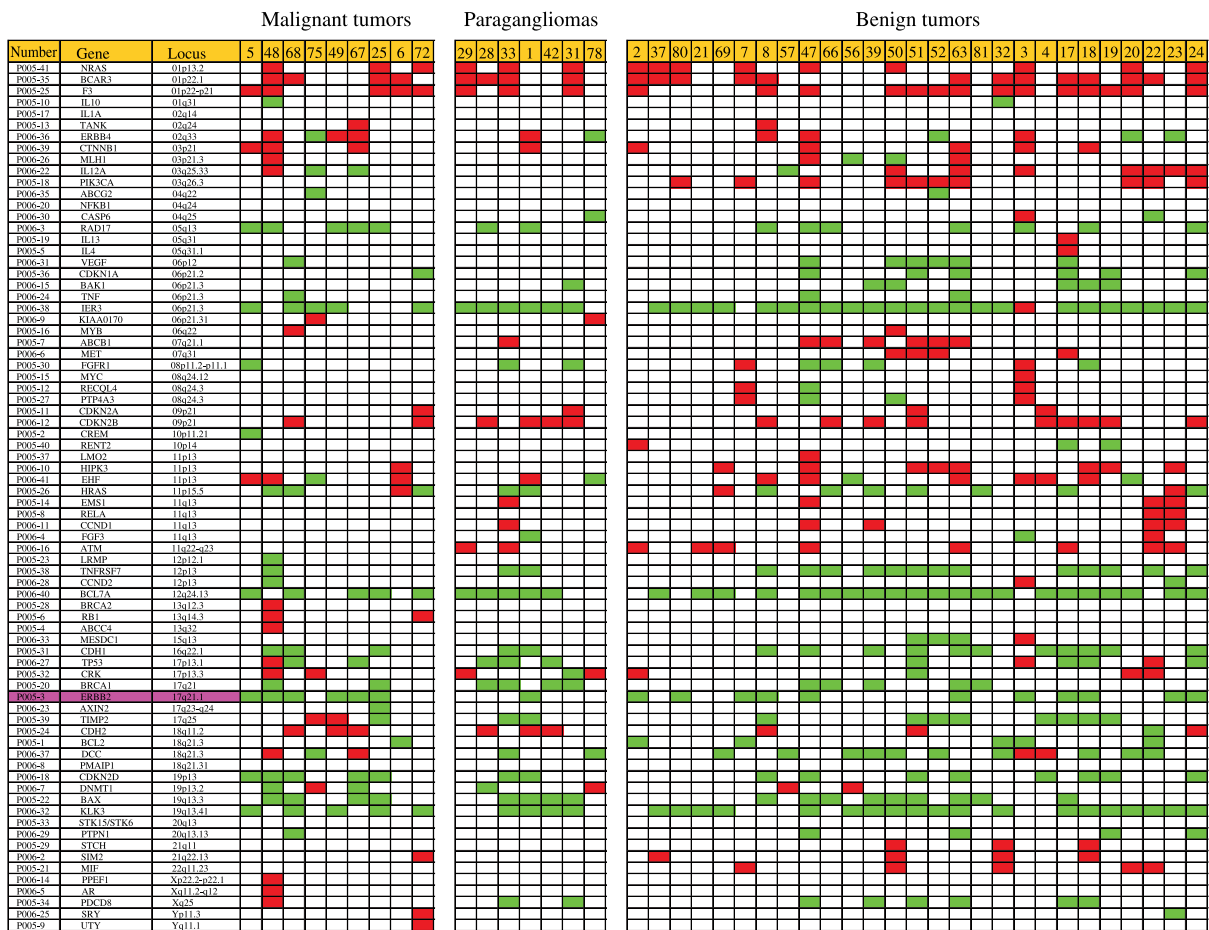
Figure 1 The copy number changes of all 43 analyzed tumors in a total of 76 genes spanning almost all chromosome arms that were included in the P005 and P006 probe mixes. Colors used: green, gain; red, loss; and white, no genetic alteration. ERBB-2 is emphasized with purple color.

Number	Gene	Locus
P005-41	NRAS	01p13.2
P005-35	BCAR3	01p22.1
P005-25	F3	01p22-p21
P005-10	IL10	01q31
P005-17	IL1A	02q14
P005-13	TANK	02q24
P006-36	ERBB4	02q33
P006-39	CTNNB1	03p21
P006-26	MLH1	03p21.3
P006-22	IL12A	03q25.33
P005-18	PIK3CA	03q26.3
P006-35	ABCG2	04q22
P006-20	NFKB1	04q24
P006-30	CASP6	04q25
P006-3	RAD17	05p13
P005-19	IL13	05q31
P005-5	IL4	05q31.1
P006-31	VEGF	06p12
P005-36	CDKN1A	06p21.2
P006-15	BAK1	06p21.3
P006-24	TNF	06p21.3
P006-38	IER3	06p21.3
P006-9	KIAA0170	06p21.31
P005-16	MYB	06q22
P005-7	ABCB1	07q21.1
P006-6	MET	07q31
P005-30	FGFR1	08p11.2-p11.1
P005-15	MYC	08q24.12
P005-12	RECQL4	08q24.3
P005-27	PTP4A3	08q24.3
P005-11	CDKN2A	09p21
P006-12	CDKN2B	09p21
P005-2	CREM	10p11.21
P005-40	RENT2	10p14
P005-37	LMO2	11p13
P006-10	HIPK3	11p13
P006-41	EHF	11p13
P005-26	HRAS	11p15.5
P005-14	EMS1	11q13
P005-8	RELA	11q13
P006-11	CCND1	11q13
P006-4	FGF3	11q13
P006-16	ATM	11q22-q23
P005-23	LRMP	12p12.1
P005-38	TNFRSF7	12p13
P006-28	CCND2	12p13
P006-40	BCL7A	12q24.13
P005-28	BRCA2	13q12.3
P005-6	RB1	13q14.3
P005-4	ABCC4	13q32
P006-33	MESDC1	15q13
P005-31	CDH1	16q22.1
P006-27	TP53	17p13.1
P005-32	CRK	17p13.3
P005-20	BRCA1	17q21
P005-3	ERBB2	17q21.1
P006-23	AXIN2	17q23-q24
P005-39	TIMP2	17q25
P005-24	CDH2	18q11.2
P005-1	BCL2	18q21.3
P006-37	DCC	18q21.3
P006-8	PMAIP1	18q21.31
P006-18	CDKN2D	19p13
P006-7	DNMT1	19p13.2
P005-22	BAX	19q13.3
P006-32	KLK3	19q13.41
P005-33	STK15/STK6	20q13
P006-29	PTPN1	20q13.13
P005-29	STCH	21q11
P006-2	SIM2	21q22.13
P005-21	MIF	22q11.23
P006-14	PPEF1	Xp22.2-p22.1
P006-5	AR	Xq11.2-q12
P005-34	PDCD8	Xq25
P006-25	SRY	Yp11.3
P005-9	UTY	Yq11.1

Malignant tumors: 5, 48, 68, 75, 49, 67, 25, 6, 72
Paragangliomas: 29, 28, 33, 1, 42, 31, 78
Benign tumors: 2, 37, 80, 21, 69, 7, 8, 57, 47, 66, 56, 39, 50, 51, 52, 63, 81, 32, 3, 4, 17, 18, 19, 20, 22, 23, 24

The deletion or the duplication of the genes represents the deletion or the duplication of the chromosome where the genes are located. In our study, chromosomes 1p, 3p, 9p, and 11p showed obvious deletion, while chromosomes 6p, 12q, 17q, and 19q showed duplication. However, there is no significant difference between malignant pheochromocytomas, paragangliomas, or benign tumors.

The most obvious difference between malignant and benign tumors is the duplication of *ERBB-2* gene locus on chromosome 17q21. In malignant tumors, gain of *ERBB-2* gene was the most common abnormality, which was detected in six out of the nine (67%) patients; while in paragangliomas and benign pheochromocytomas, only 12 out of the 34 (35%) patients showed gain of *ERBB-2* gene.

Immunohistochemistry

To study the expression of *ERBB-2* oncogene in pheochromocytomas, we further analyzed 39 out of the 43 samples by IHC analysis. Positive ERBB-2 immunoreactivity showed a diffuse intra-cytoplasmic granular staining. The frequency of cells expressing the ERBB-2 protein differed among tumors. All the malignant pheochromocytoma samples showed positive ERBB-2 immunoreactivity, among them 86% were $++$ or $+++$. None of the benign tumors showed $+++$, and 52% of the benign tumors showed

negative immunoreactivity. No immunostaining was observed in the negative controls (Fig. 2). The Kruskal–Wallis test showed significantly higher ERBB-2 oncoprotein expression in the malignant pheochromocytomas ($P = 0.0065$, K–W test; Table 3).

Discussion

The pheochromocytomas are neuronal–endocrine tumors arising from the paraganglia system found in the adrenal medulla, whereas those found along the para-vertebral and para-aortic axis, but outside the adrenals, are called paragangliomas. The pheochromocytomas can be a part of some of the hereditary syndromes, including MEN2, VHL, NF1, and paraganglioma syndrome. As the genes responsible for these hereditary cancer syndromes were identified, attention has been shifted to the identification of additional genetic alterations in sporadic pheochromocytomas, especially malignant ones (Neumann *et al*. 1993, Eisenhofer *et al*. 2004, Brouwers *et al*. 2005, Korpershoek *et al*. 2007). However, somatic genome alteration researches are very important to understand the mechanism of pheochromocytomas. Loss of chromosome 1p recently detected in pheochromocytomas (Dannenberg *et al*. 2000, Opocher *et al*. 2003, August *et al*. 2004, Aarts *et al*. 2006) was the most frequent chromosomal deletions in our study.
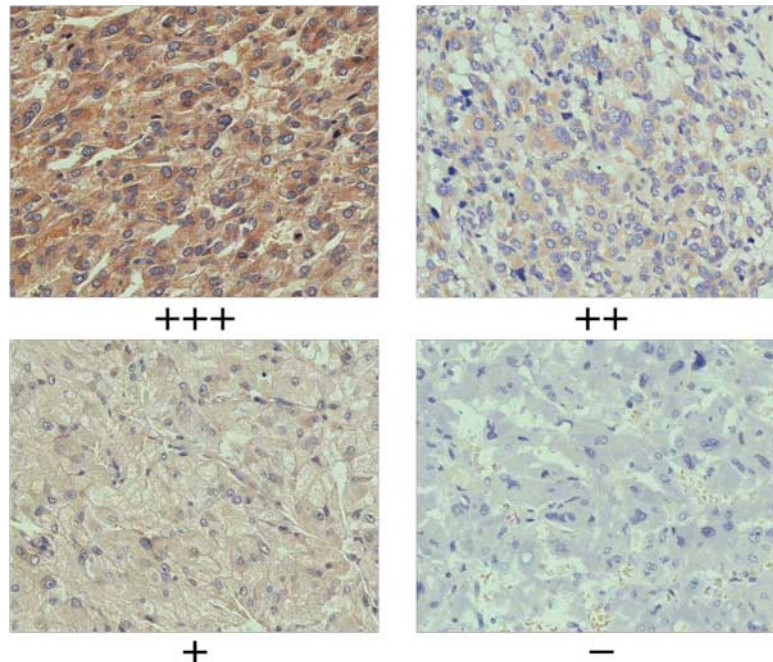

Figure 2 Strong positive staining ($+++$) of the malignant pheochromocytoma (top left), $++$ of the paraganglioma (top right), $+$ of the benign pheochromocytoma (bottom left), and negative control (bottom right). Magnification, $\times 40$.

Table 3 Immunohistochemistry study of the *ERBB-2* oncogene revealed that all malignant tumors showed positive of the ERBB-2 expression. Most of the benign tumors were negative

	Malignant (%)	Extra-adrenal (%)	Benign (%)	P value
+++	2 (29)	1 (14)	0	
++	4 (57)	2 (29)	7 (28)	
+	1 (14)	0	5 (20)	0.0065
−	0	4 (57)	13 (52)	
N	7	7	25	

The deletion occurred in all three types of pheochromocytomas with similar frequency; therefore, this may be indicative of early events in the tumorigenesis of sporadic pheochromocytoma. However, these lesions probably are of less importance in view of the progression to malignancy. Our study, revealed the frequent gain of chromosome 17q in malignant tumors, which indicated that this region may be associated with the malignancy of the disorders. These results were similar to that detected by CGH (Brinkschmidt *et al.* 2001, August *et al.* 2004).

The design of the MLPA analysis takes advantage of changes not only in chromosome but also in the genes themselves to demonstrate the relevant genetic changes in tumors. In the present study, *ERBB-2* oncogene duplication was observed most frequently in malignant tumors. *ERBB-2* oncogene, originally called NEU (neuron/glioblastoma derived oncogene homolog), was derived from the rat neuro/glioblastoma cell lines. It encodes a tumor antigen, p185, which is related to the epidermal growth factor receptor. Further studies proved that *ERBB-2* gene product is a 185 kDa glycoprotein with tyrosine kinase activity (Akiyama *et al.* 1986). Overexpression of the ERBB-2, a breast cancer marker, is associated with rapid tumor growth, increased risk of recurrence after surgery, poor response to conventional chemotherapy, and shortened survival, and this ERBB-2 overexpression has also been implicated in the neoplastic transformation of prostate cancer (Ross & Fletcher 1998, Yu *et al.* 1998). Trastuzumab (*Herceptin*) is a human monoclonal antibody that appears to block the growth signals transmitted by ERBB-2 to the nucleus and enhances the response to chemotherapeutic agents (Pietras *et al.* 1998, Slamon *et al.* 2001). Antibody against ERBB-2 has been FDA approved for clinical treatment of cancer under the brand name *Herceptin*. Some recent clinical trials have found trastuzumab reduces the risk of relapse in breast cancer patients by 50% when given in the adjuvant setting (i.e., after breast cancer surgery, before the cancer has spread any further) for 1 year (Piccart-Gebhart *et al.* 2005, Romond *et al.* 2005).

In our study, further IHC analysis confirmed that there was significant difference in malignant and benign pheochromocytomas, which indicated that *ERBB-2* oncogene may be an important gene in the malignant development of pheochromocytomas. The effects of ERBB-2 in pheochromocytomas pathogenesis may be similar to that in breast and prostate cancers.

As pheochromocytoma is a rare endocrine tumor, consequently the specimens included in our study were limited. With the increase of the tumor samples, the difference between the malignant and benign tumors may be more significant. Another limitation of our study is the difficulty in distinguishing the malignant ones from the benign, which needs long-term follow-up; and our present research has only been undertaken in a few years, there must be some malignant tumors in the 'benign' group under our present diagnosis.

In conclusion, the results from our study by MLPA showed that genomic alternation was identified in pheochromocytomas and paragangliomas, involving chromosomes 1p, 3p, 6p, 11q, 12q, 17q, and 19q. Gain of *ERBB-2* oncogene located on chromosome 17q was an obvious difference between malignancy and benign tumors. IHC analysis of ERBB-2 further confirmed that the expression of ERBB-2 in malignant tumors was much higher than that in the benign ones, whereas no ERBB-2 protein expression was found in normal tissues. The association of *ERBB-2* gene in breast cancers and prostate cancer has been widely elucidated in many studies, and the current study was only a clue to yet another possible association between ERBB-2 and pheochromocytomas, and paragangliomas, and further investigation might clarify this connection.

Acknowledgements

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