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Original Article

Mutation status of ras genes in breast cancers with overexpressed p21Ras protein

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Abstract: *Ras* gene product, p21Ras, was found to be overexpressed in human tumors. However, the subtypes of overexpressed p21Ras and mutation status are still unknown which limits the development of therapeutic antibodies targeting p21Ras. This study aims to investigate the mutation status of *ras* genes in breast cancers overexpressed p21Ras to real whether or not wild type p21Ras could be target for breast cancer therapy. Method: The expression of total p21Ras was examined immunohistochemically in normal breast tissue, usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and invasive carcinoma (IC) by monoclonal antibody (Mab) KGH-R1 which could react with three types of p21Ras. Then, the expression of p21Ras subtypes in breast cancers was examined by specific Mab for each p21Ras subtypes. Mutation status of *ras* genes of p21Ras-overexpressed breast cancer were detected by DNA sequencing. Results: There were almost no p21Ras expression in normal breast tissue, but high level of expression in breast cancers, with generally significant increasing from UDH to ADH, DCIS and IC. The expression of K-p21Ras subtype was detected in all breast cancer, however, no N-p21Ras expression was found in all breast cancer, and only 4.2% of breast cancers demonstrated H-p21Ras expression. Exon mutations were not detected in three *ras* genes in all breast cancers which overexpressed p21Ras. Conclusions: The overexpression of wild type p21Ras rather than mutant p21Ras plays a prominent role in the development of breast cancer, and wild type p21Ras is a promising target for breast cancer therapy.

Keywords: Wild type p21Ras, overexpression, mutation, breast cancers

Introduction

Breast cancer is one of the most common malignancy among female worldwide, with more than 1,000,000 new confirmed cases and about 373,000 deaths each year [1, 2]. In Asia, breast cancer is the leading malignancy and the second material cause of cancer-related deaths among female, approximately 39% of all confirmed breast cancers of the world. An estimated 231,013 women died of breast cancer in Asia in 2012, approximately 40.8% of the cancer deaths, and 7% of all deaths [3].

Surgical resection or surgery coupled with systemic chemotherapy and radiotherapy are the main approaches currently available for cancer therapy. Despite advanced progress in technologies to diagnose and treat cancers, the global burden of breast cancer continues to increase greatly [4]. Therefore, improved therapeutic approaches are needed for more efficacious control of the breast cancers. Oncogenes play an important role in carcinogenesis and progression [5], and make their products to be targets for therapeutic intervention. For example, Herceptin, a monoclonal antibody to *Her2* protein, have contributed great effects and benefits to patients suffered from breast cancers [6]. Unfortunately it is useless for the patients without HER2 overexpression. Thus, preparations of therapeutic antibodies against other oncogenes are essential strategies with great promise for breast targeting therapy.

Ras gene, are one of the earliest oncogenes discovered in human tumors, which locates in the downstream of *Her2*. *K-ras*, *H-ras*, and

N-ras are main members of *ras* gene family, which encode a protein similar to a 21 kDa protein (p21Ras). The activations of *ras* genes lead to an increase in proliferation and malignant transformation of cells [7].

Ras mutations were found in about 30% of all human cancers [8], mostly localized in codon 12, 13, 59 and 61 [9-12]. However, *ras* mutations were rare in breast cancers, it was demonstrated that *K-ras* mutations happened in about 6.5% of breast cancers [13], *N-ras* mutations are extremely rare in breast tumors [14], *H-ras* mutations was not reported in breast cancers [15]. There should be another activating mechanism of *ras* genes in breast cancers. As a confirmation for this hypothesis, elevated expression of p21Ras has been observed in about 50-60% of breast cancers [16-19], which suggested that the aberrant expression of *ras* genes may be an initial event in the development of breast cancers [20-23]. But up to now, it is unknown which subtype of p21Ras was overexpressed in breast cancers. There is still lack of evidences to answer the overexpressed p21Ras in breast cancer is mutant or wild type, which limited the development of therapeutic antibodies targeting *ras* gene. In this study p21Ras subtypes and mutation status of *ras* were investigated in breast cancers which overexpressed p21Ras.

Materials and methods

Tissue collection

57 cases of normal breast tissues (with a 5-cm distance from the tumor margin), 55 of breast usual ductal hyperplasia (UDH), 33 of atypical ductal hyperplasia (ADH), 35 of ductal carcinoma in situ (DCIS) and 84 invasive ductal carcinoma (IC) were collected between January 2014 and April 2015 in the Kunming General Hospital. All patients who had undergone surgery or biopsy of both primary tumors and adjacent tissues were randomly selected. The tissues were fixed in 10% buffered formalin and embedded in paraffin and diagnosed by pathologists.

Of the 84 breast cancers, 12 samples were well-differentiated, 67 were middle-differentiated, and 5 were poorly-differentiated. All patients were female and with ages from 29 to 78. None of the patients were claimed familial breast cancer history.

Antibodies

The monoclonal antibody KGH-R1 against all three p21Ras (K-p21Ras, H-p21Ras and N-p21Ras) was prepared by our laboratory [24]. The monoclonal antibody 60309-1-Ig to K-p21Ras was purchased from Proteintech Group (Chicago, IL), monoclonal antibody sc-31 to N-p21Ras and monoclonal antibody sc-29 to H-p21Ras were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Tissue microarray construction

The areas containing cancer tissue were annotated on the HE slides and all tumor diagnoses were confirmed by 2 experienced pathologists. TMA was constructed using a tissue array instrument (Boyikang, Beijing, China). Four-millimeter cores that contained cancer tissue were removed from the selected area (region of interest) using a needle punch. Subsequently, these cores were embedded in beforehand arranged recipient paraffin blocks with a precisely spaced 25-hole array pattern.

Immunohistochemical staining

Paraffin-embedded tissues in tissue microarray block were subjected to immunohistochemical staining. Briefly, 4 um tissue microarray sections were deparaffinized in xylene, hydrated in descending concentrations of ethanol (2×100% for 3 min, 95% for 3 min and 85% for 3 min) and rinsed with double-distilled water for three times. The slides were treated by autoclave in citrate acid buffer (0.01 M PH 6.0) to unmask antigens and then exposed to 3% H_2O_2 for 10 minutes to block the endogenous peroxidase reactivity, followed by rinsing with double-distilled water. To avert unspecific staining, 10% BSA was used to block the sections for 40min at 37°C. The sections were incubated with p21Ras monoclonal antibody overnight at 4°C, then rinsed with 0.01 mol/L PBS. Subsequently, it was exposed to horse radish peroxidase secondary antibody (ZSGB-BIO, Beijing, China) for 30 minutes, and washed with PBS three times. Tissue sections were visualized by diaminobenzidine tetrahydrochloride (DAB) for 5-7 minutes. Finally, all slides were counterstained with hematoxylin, dehydrated and mounted with neutral resin.

The result of immunohistochemistry was evaluated by histological score (Hscore) [25]. 300

Table 1. Primers for sequencing of RAS exons

	Target fragment	Primer	Sequences (5'-3')	Product length (bp)	Annealing temperature (°C)
K-ras	Exon 2	Sense	TTATTATAAGGCCTGCTG	185	52.3
		Antisense	TGTATCAAAGAATGGTCC		
	Exon 3	Sense	GTGTTTCTCCCTTCTCAG	274	50.0
		Antisense	GGCATTAGCAAAGACTCA		
	Exon 4	Sense	TGTTACTAATGACTGTGCTA	255	46.1
		Antisense	TGTTACTAATGACTGTGCTA		
	Exon 5	Sense	ACATGGCTTTCCCAGTAA	263	50.0
		Antisense	GTTGCCACCTTGTTACCT		
H-ras	Exon 2	Sense	AGACCCTGTAGGAGGACCC	187	58.1
		Antisense	CTGCTGGCACCTGGAC		
	Exon 3	Sense	CACGGAAGGTCCTGAGGGG	276	61.0
		Antisense	GCCTGGCCCCACCTGTG		
	Exon 4	Sense	CTCTCGCTTTCCACCTCT	207	58.6
		Antisense	AGCTGTGGGGTGGAGA		
	Exon 5	Sense	GGCAGGCGGCCACAGG	215	61.9
		Antisense	ATCCGGTGGGCGTGGC		
N-ras	Exon 2	Sense	AATTAACCCTGATTACTGG	217	51.9
		Antisense	TAAAGATGATCCGACAAG		
	Exon 3	Sense	TAACCTTGGCAATAGCAT	339	52.3
		Antisense	TAACCTCATTTCCCCATA		
	Exon 4	Sense	CATGAGCCACTGTACCCA	255	54.9
		Antisense	TTGCACAAATGCTGAAAG		
	Exon 5	Sense	GAGATACAAATGCAAGAG	286	50.9
		Antisense	ATAACACCAGCACTCCT		

*Exon 1 of *ras* not encode the protein (*K-ras*, *H-ras* and *N-ras* DNA sequence in GenBank AF493917, AF493916, AF493919).

cells were counted in every component on every slide, the intensity of specific staining was characterized as: 0 = none (no staining cells), 1 = weak (primrose yellow cells), 2 = medium (yellow cells), 3 = strong (tawny cells). A mean percentage of positive cells was determined in at least 3 areas at 40× magnifications and assigned from 0% to 100%.

DNA extraction

We used 10 μm-thick formalin fixed and paraffin-embedded (FFPE) serial sections for DNA extraction. Genomic DNA was extracted from the tissue by means of the QIAamp DNA FFPE Tissue Kit according to the QIAamp DNA FFPE Tissue handbook. The purity of the extracted DNA was tested using an ultraviolet spectrophotometer, and DNA samples were stored at -20°C for further analysis.

PCR amplification

The primers for *K-ras*, *N-ras* and *H-ras* amplication were designed by Primer 5.0 and oligo software. Primer sequences, amplified product size, and annealing temperatures are listed in **Table 1**. Each 25 μl PCR reaction mixture contained 2.5 μl of 10x buffer, 2 μl dNTP (10 mmol l^{-1}), 1 μl Taq DNA polymerase, 1 μl of forward primer and 1 μl of reverse primer (10 μmol/L), DNA template (at least 800 ng) and sterile deionised water. The PCR conditions for PCR reactions were initial denaturation at 95°C for 4 min, 30 cycles of 95°C for 30 s, suited annealing temperature for 30 s, amplification at 72°C for 30 s, and a final extension at 72°C for 10 min. Subsequently, the PCR amplification products were separated by 1% agarose gel electrophoresis (AGE) for 40 min and imaged using the imaging system.

DNA sequence analysis

20 μL PCR products of samples were sequenced in Hua Da Gene (Beijing, China) with up-stream or down-stream primers. Sequences obtained were aligned with nucleic acids and amino acid sequence of normal human RAS, and analyzed by Invitrogen Align X and Chromas software.

Statistical analysis

The data were collected and analyzed by the SPSS software package, standard version 22.0. Quantitative parameters were analyzed by mean and standard deviation. The differences of all tests were two-side, and analyzed by chi-squared distribution (χ^2). A value of

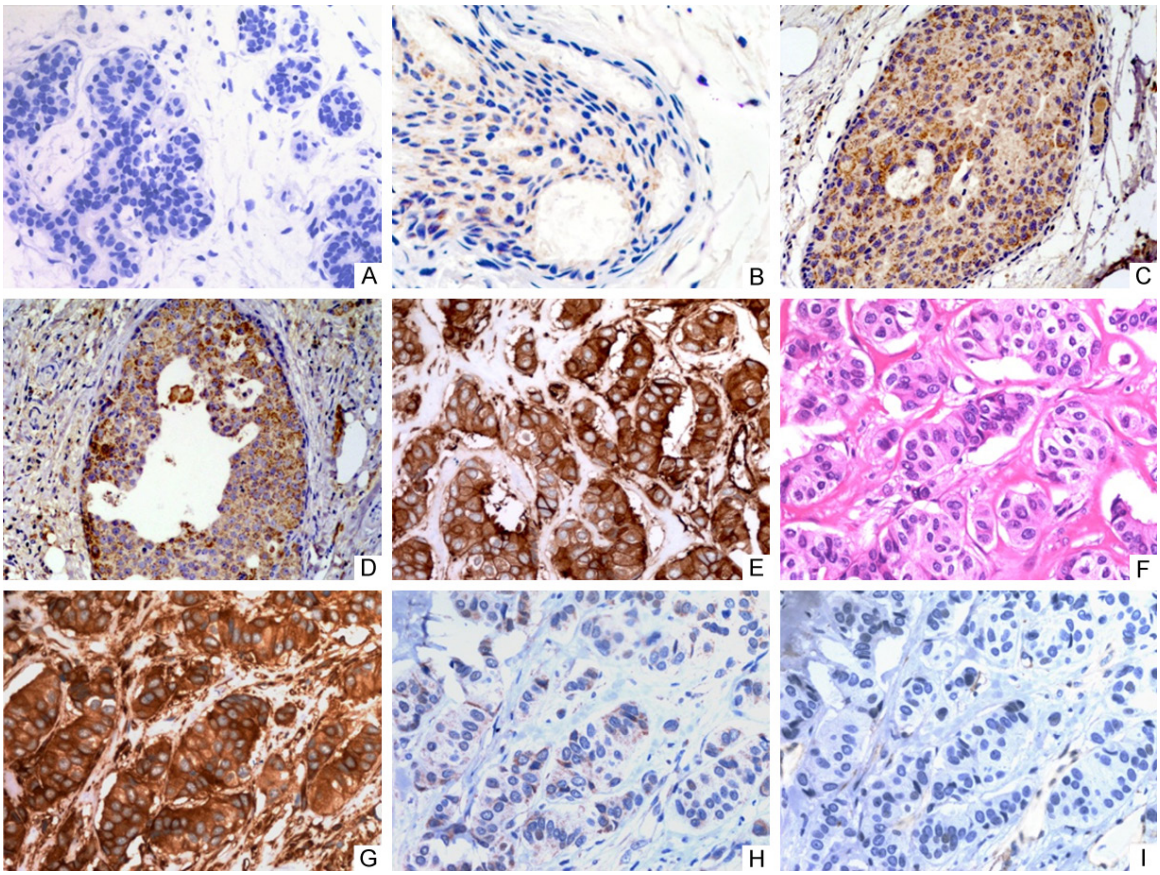

Figure 1. Expression of total p21Ras and p21Ras subtypes detected by immunohistochemistry, 200×. (A-E) Total p21Ras expression detected by Mab KGHR-1; there were no staining in normal breast tissue (A), weak staining in UDH (B), medium staining in ADH (C), strong staining in DCIS (D) and IC (E). (F-I) Expression detection of p21Ras subtypes in same case of IC; HE staining (F), strong stain of K-p21Ras (G), weak staining of H-p21Ras (H), weak staining of N-p21Ras (I).

Table 2. Comparative analysis of the immunohistochemical profile of p21Ras expression in breast lesions

Group	n	Positive (n)	The percentage of positive cells	HSCORE
Normal	57	12	1.53±3.41	0.87±2.29
UDH	55	27	11.36±17.50	10.77±18.66
ADH	33	19	26.96±27.54	31.58±41.27
DCIS	35	22	37.20±33.22	54.53±58.77
IC	84	67	58.16±49.65	112.28±103.85

$P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of p21Ras

12 of normal breast tissues, 27 of UDH, 19 of ADH, 22 of DCIS, and 67 of IC were employed to detect total p21Ras expression using MAb KGH-R1 and immunohistochemical assay. The positive products were localized in cytoplasm which represented p21Ras expression (**Figure 1**). Heterogeneous distribution of p21Ras expression in different characteristics of breast lesions was found. There was almost no p21Ras expression in normal breast tissue (**Figure 1A**). Expression of p21Ras increased gradually from UDH to ADH, DCIS and IC, and strong staining was showed in IC (**Figure 1B-E**). With the progression of the disease, the positive cell proportion and positive intensity were increased.

We used Hscore to evaluate the expression of p21Ras. The mean Hsores were 0.87±2.29, 10.77±18.66, 31.58±41.27, 54.53±58.77, 112.28±103.85 in normal breast tissue, UDH, ADH, DCIS and IC, respectively. Comparative analysis of the immunohistochemical profile of

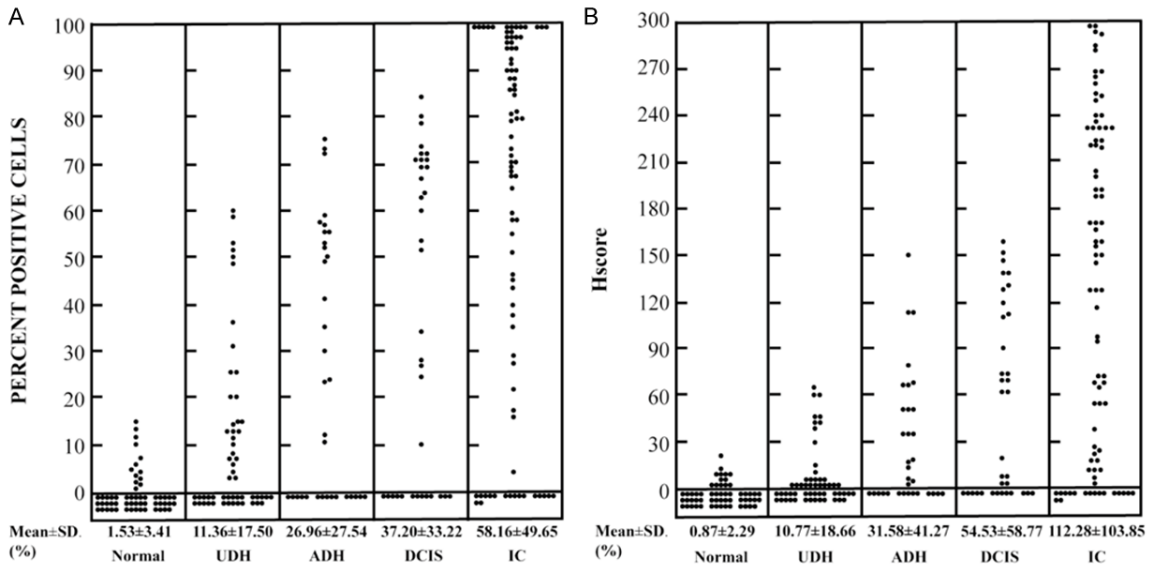

Figure 2. The percentage of positive cells (A) and Hscores (B) of p21Ras expression for each cases. Normal: normal breast tissues, UDH: usual ductal hyperplasia, ADH: atypical ductal hyperplasia, DCIS: ductal carcinoma in situ, IC: invasive carcinomas.

Table 3. Correlation of Ras p21 Expression With Clinicopathologic Features of breast cancer Patients

Item	Case	HSCORE	*P* value
Age			
≤50	56	97.55±105.26	>0.05
>50	28	104.23±99.12	
Lymph node metastasis			
Yes	18	89.42±97.65	>0.05
No	66	98.30±105.12	
ER			
+	44	104.40±101.28	>0.05
-	27	110.14±102.67	
PR			
+	39	95.76±92.90	>0.05
-	32	108.84±104.52	

*Spearman grade correlation analysis; Only 71 of the 84 patients had complete ER immunohistochemical data.

p21Ras expression showed that the Hscores and percentage of positive cells were increased successively from breast hyperplasia to cancer (**Table 2**). The percentage of positive cells and Hscores of each case were in **Figure 2**.

Correlation between p21Ras expression and clinicopathologic variables

We compared the age, histological type, differentiation, tumor size, ER, PR expression and lymph node metastasis with Hscore and the percentage of positive cells in 84 breast cancer patients. The correlation between p21Ras expression and clinicopathologic variables of breast cancers is listed (**Table 3**). No correlation was observed between Hscore and clinicopathologic features mentioned above (P>0.05).

Expression of p21Ras subtypes

Anti K-p21Ras Mab, anti H-p21ras Mab and anti N-p21ras Mab were employed to detect the expression p21Ras subtypes in 24 cases of breast cancers by immunohistochemistry (**Figure 1G-I**). All 24 breast cancer tested were demonstrated to express K-p21Ras, and only one case of breast cancer (4.2%) expressed H-p21Ras, and no N-p21Ras expression was found in all breast cancer tissues.

PCR amplification and DNA sequence

2-5 exons of *K-ras*, 2-5 exons of *H-ras* and 2-5 exons of *N-ras* were amplified successfully from 24 cases of breast cancers by our designed primers. Each PCR product was confirmed in 1% agarose gel electrophoresis with expected sizes of 185, 274, 255, 263, 217, 339, 255, 286, 187, 276, 207 and 215, respectively (**Figure 3A**). None of mutations was identified in all exons of three *ras* genes in all tested breast cancer tissues (**Figure 3B**, **3C**).

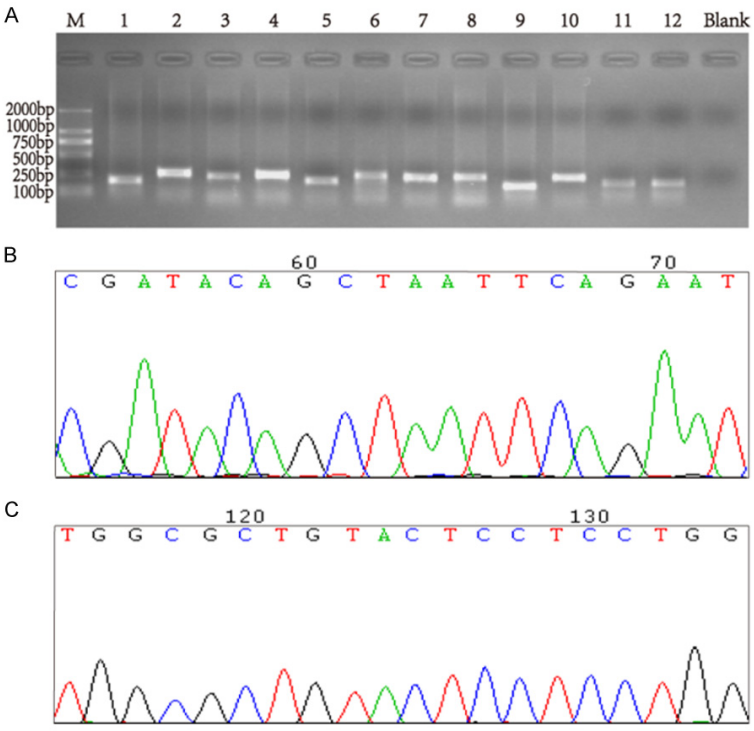

Figure 3. A: Agarose gel electrophoresis showed the size of ras exons amplicons in breast cancers, K-ras 2-5 exon (lane 1 to 4), N-ras 2-5 exon (lane 5 to8), H-ras 2-5 exon (lane 9 to 12). B: DNA sequencing for K-ras exon 2 (no mutation). C: DNA sequencing for H-ras exon 2 (no mutation).

Discussion

Amplification and overexpression have been identified to be main mechanisms for oncogenes activation which lead to tumorigenesis and progression. Overexpression of c-*myc* occurs in up to 42% of pancreatic ductal adenocarcinoma (PDAC), activated c-*myc* signaling can be sufficient to enable carcinogenesis of PDAC [26, 27]. In addition, *c-Jun* is frequently overexpressed in human cancers [28]. The human epidermal growth factor receptor 2 (*Her2*) overexpressed in 20-25% of breast carcinomas [29]. Overexpression of *bcl-2* was found in about 75% of breast cancers [30]. Thus, oncogene products become potential targets for tumor therapy, and now, the target therapy to oncogene proteins is a significantly developing aspect. For instance, Trastuzumab, a humanized IgG1 monoclonal antibody against *Her2* proteins showed good clinical efficacy in breast cancers which overexpressed *Her2* proteins [31]. The Aurora-A inhibitors, MLN8054, which disrupt the Aurora-A/N-Myc complex and promote degradation of N-Myc, have a more rapid impact on N-Myc levels. Treatment with MLN8054 broadly inhibits N-Myc dependent transcription in vivo [32]. ABT-737 (a pre-clinical lead compound), its orally available counterpart ABT-263 (Navitoclax) and GDC-0199/ABT-199 (Venetoclax), three BH3 mimetics against *Bcl-2* protein have been shown to have high antitumoral function [30].

p21Ras overexpression was found in many human tumors, however, so far no drugs which directly target p21Ras been developed successfully. More recently, we prepared a novel anti-p21Ras MAb, KGHR-1, using wild type p21Ras as immunogen, and found it could recognize and react with three type of p21Ras (H-p21Ras, N-p21Ras and K-p21Ras) [24]. The single chain antibody (scFv) developed from the KGHR-1 MAb achieved significant antitumoral efficacy in vitro and in vivo [33]. Here KGHR-1 Mab was used to evaluate total p21Ras expression in benign and malignant breast tissues by immunohistochemical assays, and found that there were no p21Ras expression in normal breast tissues, with generally increasing expression in UDH, ADH, DCIS, and IC, respectively, and IC demonstrated high level of overexpression of p21Ras. This data confirmed that p21Ras overexpression is an early event, and play an important role in the development of breast cancers.

Subtype detection assay showed that K-p21Ras was overexpressed in all breast cancers, H-p21Ras in only 4.2% of cases, and N-p21Ras in none of cases. Our data indicated that K-p21Ras rather than N-p21Ras and H-p21Ras is deeply involved in breast cancer development.

Our DNA direct sequence analysis revealed that there were no *ras* mutations in all breast cancer tissues overexpressed p21Ras, which means that the overexpressed p21Ras was

wild type instead of mutant type. Thus, in breast carcinogenesis the overexpression of wild type p21Ras is more prominent mechanism of *ras* activation than overexpression of mutant p21Ras, and the antibody drugs target wild type p21Ras may have better prospects for breast cancer therapy.

Although the activation mechanism of *ras* mutation is clear [8], how the wild type p21Ras overexpression induce proliferation and malignant transformation remains unknown. It is reported that wild type p21Ras can activate JAK2, either alone or together with the Wnt pathway, to stimulate cell proliferation and transformation [34]. On the other hand, we speculate that accumulation of wild type p21Ras may lead to the excessive GTP-bound active forms which cannot be completely hydrolyzed, finally stimulate cell proliferation and tumorigenesis.

In conclusion, we detected the expression level of p21Ras in benign and malignant breast lesions, as well as the p21Ras subtypes and mutation status of *ras* gene in breast cancers. The results suggested that the overexpression of wild type p21Ras especially K-p21Ras plays an prominent role in the development of breast cancers, wild type p21Ras is a promising target for breast cancer therapy and the antibody drug to wild type p21Ras should be developed.

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Disclosure of conflict of interest

None.

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