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Nuclear localization of GLI1 and elevated expression of FOXC2 in breast cancer is associated with the basal-like phenotype

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Summary. Aberrant sonic hedgehog (SHH)/glioma-associated oncogene (GLI) signaling has been shown in the development of many tumors. The aims of the present study are to determine the expression of two SHH signaling molecules, the glioma-associated oncogene homolog 1 (GLI1) and forkhead box C2 (FOXC2), in invasive breast cancers (IBC), to evaluate their association with clinicopathological parameters, and to determine their prognostic significance in breast cancer patients. Expression of GLI1 and FOXC2 were assessed by immunohistochemical analysis of a tissue microarray containing 262 unselected IBC cases. A statistical analysis was performed to assess the correlation of GLI1 and FOXC2 expression with the patients' clinicopathological parameters, postoperative survival rate, and molecular subtypes. Immunoreactivity of GLI1 and FOXC2 was observed in 84% and 75% of all breast cancer tissues, respectively. There was a significant correlation between nuclear FOXC2 and GLI1 expressions in these breast cancers, which was associated with estrogen receptor (ER) negativity. Furthermore, there was a significant association between nuclear expression of GLI1 and FOXC2 and a basal-like breast cancer phenotype. Patients with nuclear GLI1 or FOXC2-expressing tumors had a significantly shorter survival time than those without nuclear FOXC2 or GLI1 expression. Multivariate analysis showed that nuclear GLI1 or FOXC2 expression was an independent factor for predicting the prognosis of basal-like breast cancer. In conclusion, there was a significant correlation between expression of nuclear GLI1 or FOXC2 and human breast cancer. More specifically, elevated levels of these proteins were associated with the basal-like breast cancer phenotype and with a poor rate of disease-free survival. These data suggest that GLI1 and FOXC2 are involved in tumorigenesis and that they may be useful as diagnostic and therapeutic targets for human basal-like breast cancers. Additional studies are warranted to better understand the biological significance of GLI1 and FOXC2, to further refine statistics related to patient prognosis, and to optimize treatment of patients with basal-like breast cancer.

Key words: SHH, Sonic Hedgehog, GLI1, Glioma-associated oncogene homolog 1, FOXC2, Forkhead box C2, IBC, Invasive breast cancers

Introduction

The Sonic Hedgehog (SHH)/Glioma-associated oncogene (GLI) signaling network is one of the most important signal transduction systems that provide a central role in the regulation of many developmental and physiological processes. Aberrations in the SHH/GLI cascade can lead to the development of a wide variety of aggressive and metastatic cancers (Watkins et al., 2003; Karhadkar et al., 2004). GLI1, a member of the GLI

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Abbreviations: SHH, Sonic Hedgehog; GLI1, glioma-associated oncogene homolog 1, FOXC2, Forkhead-Box C2; IBC, invasive breast cancers; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; EMT, epithelial mesenchymal transition; BLBC, basal-like breast cancers; CK5/6, cytokeratins 5/6; EGFR, epidermal growth factor receptor; CK14, cytokeratin 14; H&E, hematoxylin & eosin; NPI, Nottingham prognostic index; OS, overall survival; DFS, disease-free survival; TMA, Tissue Microarray; FISH, fluorescent *in situ* hybridization

family, is a strong positive activator of downstream target genes and is itself a representative target gene of the SHH signaling cascade. GLI1 can also be up-regulated by both Sonic Hedgehog signaling and hedgehog-independent mechanisms, through either Ras or TGF-beta stimulation (Lee et al., 1997; Lauth and Toftgard, 2007). GLI1 is overexpressed in various types of human tumors, such as skin basal cell carcinoma (Dahmane et al., 1997), lung cancer (Watkins et al., 2003), gastric cancer (Ma et al., 2005), pancreatic cancer (Thayer et al., 2003), esophageal cancer (Ma et al., 2006), and breast cancer (Xu et al., 2010; Zhao et al.,2010). Several studies have found associations between the expression of GLI1 and tumor invasiveness, the status of lymph node metastasis, and unfavorable survival (Mori et al., 2006; Yoo et al., 2008).

Downstream targets of GLI1 signaling include both oncogenic products and transcription factors, such as the Forkhead-Box (FOX) factors. FOX protein family members constitute a large family of transcription factors that are implicated in both embryonic development and adult tissue homeostasis, as they regulate the key activities of cell growth, proliferation, differentiation, longevity and transformation (Sano et al., 2010). Recent analyses on GLI1 revealed potential involvement of some FOX protein family members in tumor development processes (Katoh et al., 2009). We also chose to focus more in-depth research on FOXC2, which specifically promotes mesenchymal differentiation during an epithelial mesenchymal transition (EMT), as a recent study shows that human FOXC2 is strongly expressed in highly aggressive basal-like breast cancers (BLBC) and is responsible for invasion and metastasis (Mani et al., 2007).

BLBC make up about 15% of all human breast cancers. BLBC, a molecular subtype, expresses genes that are characteristic of the basal/myoepithelial cells of the normal mammary gland. BLBC is identified by immunohistochemical staining. It is positive for cytokeratins 5/6 (CK5/6) and/or epidermal growth factor receptor (EGFR) and/or P-cadherin and/or cytokeratin 14(CK14), yet negative for the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2(HER-2). BLBC encompass 60% to 90% of triple-negative breast cancers; they are associated with high histologic grade, aggressive clinical behavior, a high rate of metastasis and an unfavorable patient survival rate (Perou et al., 2000; Sorlie et al., 2003; Nielsen et al., 2004; Livasy et al., 2006). Whereas the presence of ER and HER2 guide the treatment of luminal and HER2 breast cancers, respectively, chemotherapy is still the only systemic therapy modality for treating BLBC. Unfortunately, standard chemotherapy in BLBC patients is associated with a high rate of either local or systemic relapse (Cheang et al., 2008; Parikh et al., 2008).

Several studies show that there is a potential role for GLI1 or FOXC2 in the oncogenesis of breast cancers (Mani et al., 2007; Xu et al., 2010; Zhao et al.,2010). However, the association and the biological significance of GLI1 and FOXC2 co-expression in human breast cancer have still not been well-clarified. In this study, we analyzed GLI1 and FOXC2 expression using a relatively large number of breast carcinomas available as an array and revealed for the first time that there was indeed not only a significant correlation between nuclear GLI1 and FOXC2 expression in breast cancer, but that elevated levels of nuclear GLI1 and FOXC2 protein were specifically associated with the basal-like phenotype and could be a prognostic marker for breast cancer.

Materials and methods

Patients

Samples of 262 breast cancer tissues and 20 normal breast tissues were derived from patients that underwent primary surgery for breast cancer at the Department of Surgery in Tongji Hospital (Huazhong University of Science and Technology, Wuhan, China) from 2000 to 2003. All the selected breast cancer tissues with the following inclusion criteria: no history of any other type of malignant tumor, without neoadjuvant therapy prior to surgery. Patients with only carcinoma in situ were excluded from this study. All patients gave informed consent for analysis of their tissue for research purposes. This study was approved by the Institutional Review Board for analysis of human tissues. The patients ranged in age from 31 to 77 years (median age, 56.2 years). All these selected untreated breast cancer patients underwent axillary node excision combined with wide local excision or mastectomy. Histological examination of the excised breast tissue was carried out following hematoxylin & eosin (H&E) staining of paraffin-embedded sections. Invasive breast cancers (IBC) were routinely divided into categories: invasive ductal carcinoma, invasive lobular carcinoma, and others such as medullary carcinoma and mucinous carcinoma. For each patient, the grade of IBC was classified as grade I (low), grade II (moderate), or grade III (high) according to observations of cell mitosis, tubule formation and nuclear pleomorphism. The presence or absence of lymph node metastases was noted. The tumor stage was determined according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual. The Nottingham prognostic index (NPI) was used to determine patient prognosis following surgery for breast cancer. Its value is calculated using three pathological criteria: the size of the lesion(S); the number of involved lymph nodes(N); and the grade of the tumour(G). The index is calculated using the formula: NPI= (0.2xS)+N+G. Other clinical and pathologic parameters were obtained from the hospital pathology reports (Table 1).

Adjuvant systemic chemotherapy and/or adjuvant hormone therapy were administered in accordance with standard clinical practices. Patients with an NPI score ≤3.4 received no adjuvant therapy, those with NPI score

>3.4 received tamoxifen if ER positive or classical cyclophosphamide, methotrexate and 5-fluorouracil if ER negative and fit enough to tolerate chemotherapy.

Follow-up

Follow-up data retrieved from the clinical record ranged from 3-72 months post-surgery (median, 48.2 months). Each patient's overall survival (OS) is calculated as the period from the date of surgery until the date of death, while disease-free survival (DFS) is the period from surgery to the date of metastasis. Death from a cause other than cancer relapse or survival at the end of the observation period was considered to be a censoring event for this study. We lost track of 7 patients during the observation period, so follow-up data was available for 255 of the original 262 patients. At the end of our follow-up period, 192 patients were found to be disease-free, while 63 breast cancer patients relapsed. 59 of these subsequently died. The 5-year survival rate was 77.5%.

Tissue microarray (TMA)

The formalin-fixed, paraffin-embedded blocks were retrieved, plus their matching HE-stained slides were screened for representative tumor regions. A tissue microarray was constructed with Tissue Microarrayer (Beecher Instruments, Silver Springs, MD, USA), as described (Kononen et al., 1998). The tissue microarray included 262 primary breast samples representing different histological types and grades. Each tumor was sampled in duplicate from the chosen representative areas, using a 0.6-mm punch, so there were a total of 524 tissue cores used in the array.

Immunohistochemistry

Immunohistochemical analysis of the TMA was carried out according to the manufacturer's instructions. Briefly, the sections were first deparaffinized in xylene and subsequently rehydrated through a graded ethanol series, ending in deionized water. Peroxidase blocking solution was used to block endogenous peroxidases. Antigen retrieval was performed by heating for 1.5 minutes in a pressure cooker, using a 0.01M citrate buffer (pH 6.0). Immunohistochemistry was performed following the Envision method. GLI1 antibody (1:100 dilution, Santa Cruz sc-20687) and FOXC2 antibody (1:200 dilution, ab65141, Abcam) were applied and left overnight at 4°C. As a negative control, pre-immune serum was substituted for the primary antibody. All sections were rinsed in PBS three times, 5 minutes each. Additional immunohistochemistry was performed with antibodies against CK5/6 (clone D5/16B4, 1:50 dilution, DAKO), CK14 (LL002, 1:60 dilution, DAKO), EGFR (EGFR.113, 1:200 dilution, DAKO), P-cadherin (clone 56, 1:250 dilution, DAKO), ER (1D5, 1:150 dilution, DAKO), PR (PgR636, 1:125 dilution, DAKO), HER2 (Polyclone, 1:175 dilution, DAKO), and Ki67 (MIB-1, 1:50 dilution, DAKO). Following the primary antibody rinse, the secondary antibody (Envison, Anti-Mouse/Rabbit-HRP, DAKO) was applied and incubated for 30 minutes at RT, followed by three PBS rinses of 5 minutes each. Finally, the antibody-treated sections were developed by applying 3,3'-diaminobenzidine (DAB) for 5 minutes at RT, then counterstaining with hematoxylin. After staining, the sections were dehydrated by passing them through a graded series of ethanol baths, followed by xylene, and then cover-slipped.

Evaluation of immunohistochemical staining

Immunohistochemistry results were considered ER and PR positive if the tissue was scored with more than 10% positive cells. At least 10% of tumour cells with membranous/cytoplasmic reactivity for cytokeratin (CK) 5/6, CK14, P-cadherin, membranous reactivity for epidermal growth factor receptor (EGFR) were considered positive. HER-2/neu was scored on a scale from 0 to 3+, based on an interpretation of the staining present (0 and 1+ were classified as negative, 3+ as positive). Any tumor samples with an intermediate HER-2/neu staining score of 2+ were further assayed by fluorescent in situ hybridization (FISH) to determine the presence of HER-2 amplification. All samples that had intense, complete, membranous staining in 30% of invasive tumor cells as determined by IHC or FISH-confirmed presence of HER-2 gene amplification were considered HER-2 amplification positive. These breast cancers were then divided into subtypes that included luminal A (positive for ER and/or PR and negative for HER-2), luminal B (positive for ER and/or PR and positive for HER-2), HER-2 overexpression (negative for ER and PR and positive for HER-2), and basal-like (triple-negative for ER, PR, and HER-2; yet positive for CK5/6 and/or EGFR and/or P-cadherin and/or CK14).

Immunohistochemical staining of GLI1 and FOXC2 were evaluated and scored by a pathologist who was blinded to patients' clinical information. The expressions of GLI1 and FOXC2 were evaluated for both cytoplasmic and nuclear presence. The expressions of GLI1 and FOXC2 were scored as intensity of staining: 0 (no staining), 1 (weak/moderate staining) or 2 (intense staining). Gli1 nuclear overexpression was identified for intensity cases with staining intensity of 2+. Nuclear FOXC2 positivity was identified for cases with staining of 1+ or 2+.

Statistical analysis

For all IBC cases, immunoreactivity of the GLI1 and FOXC2 proteins present in the tissue samples was compared with the patients' various clinicopathologic characteristics. The Chi-square and Fisher's exact tests were used to assess the differences in immunohistochemical staining levels between or among different groups. Spearman's rank correlation was applied, to

determine the correlation between the immunoreactions to GLI1 and FOXC2 proteins.

The prognostic values of GLI1 and FOXC2 on disease-free survival (DFS) and overall survival (OS) of primary breast cancer patients was determined for all patients (including deaths). Univariate survival curves were generated by employing the Kaplan and Meier method. The significance of observed differences was assessed using the log-rank test. The Cox regression model was also used to examine several combinations and interactions of different prognostic factors in a multivariate analysis; however, only parameters that achieved statistical significance for disease-free survival or overall survival in the log-rank test were included. A determination of statistical significance for observed differences was set at $P<0.05$. All data were analyzed using SPSS statistical software.

Results

GLI1 protein and FOXC2 protein expression in breast cancer tissue

By immunohistochemistry staining, all 20 normal breast tissues showed negative or weak GLI1 expression in the cytoplasm and/or the nucleus; however, GLI1 protein expression was detectable in 84% (220/262) of the breast cancers. We found that GLI1 protein in these breast cancer cells had a subcellular localization of either cytoplasmic staining only, or nuclear with or without cytoplasmic staining. As nuclear localization of GLI1 is likely to be a better indicator of GLI1 transcriptional activity than cytoplasmic GLI1, we focused our analyses on nuclear staining. The nuclear immunoreactivity pattern ranged from low immunoreactivity to high immunoreactivity. In 33.6% (88/262) of the cancers, there was high nuclear immunoreactivity of GLI1. The FOXC2 protein was not detected in most normal breast cells, except in a small percentage of basal epithelial cells. In contrast, FOXC2 protein expression was detectable in 75% (196/262) of breast cancers. Subcellular localization of FOXC2 protein in the breast cancer cells was also cytoplasmic staining only, or nuclear with or without cytoplasmic staining. In the cancer cells, FOXC2 immunoreactivity ranged from cytoplasmic staining, and/or nuclear staining with or without perinuclear staining. In 17.1% (45/262), we observed nuclear localization with or without cytoplasmic expression (Fig. 1 and Table 1).

GLI1 and FOXC2 expression correlated with clinicopathologic parameters

We correlated either the GLI1 or FOXC2 expression data to clinicopathological characteristics such as age, size, histological grade, lymphovascular invasion, lymph node status, NPI, ER, PR, HER-2 amplification and the rate of proliferation (Index of MIB-1). High nuclear immunoreactivity of GLI1 was found to be significantly correlated with ER negativity ($P=0.031$). Independently, FOXC2 nuclear expression was also directly associated with ER negativity ($P=0.019$). There were no significant correlations found between GLI1 or FOXC2 expression and a patients' age, histological type, clinical stage, NPI, vascular invasion, or the rate of proliferation ($P>0.05$) (Table 1).

FOXC2 protein expression correlated with GLI1 nuclear overexpression

All these breast cancer cases having nuclear FOXC2 expression were seen in conjunction with nuclear GLI1 overexpression. None of the tumors with negative cytoplasmic or nuclear FOXC2 expression had nuclear GLI1 overexpression. Both nuclear GLI1 overexpression and nuclear FOXC2 expression was detectable in 15% (40/262) of breast cancers (Table 1). Indeed, there was a significant correlation between FOXC2 nuclear expression and GLI1 nuclear overexpression in breast cancers ($P<0.001$).

GLI1 and FOXC2 expression correlated with the Basal-Like Breast Cancer (BLBC) subtype

We independently compared the expression of GLI1 and FOXC2 with the diagnosed molecular subtypes for breast cancer (Table 1). The illustration in Figure 1 shows GLI1 and FOXC2 expression in the four representative molecular breast cancer subtypes (these are: luminal A, luminal B, HER-2-overexpressing, and basal-like subtypes). Interestingly, we found that there was a significant association between nuclear GLI1 overexpression or nuclear FOXC2 expression and the basal-like phenotype markers such as P-cadherin ($P=0.091$, $P=0.033$), Cytokeratin 5/6 ($P=0.003$, $P<0.001$), and Cytokeratin 14 ($P<0.001$, $P<0.001$).

Survival analysis

The results of survival analyses are summarized in Table 2. In a univariate analysis, Nuclear GLI1 overexpression or FOXC2 expression were significantly correlated with a short period of OS ($P=0.006$ and $P=0.007$, respectively) and DFS ($P=0.003$ and $P=0.001$, respectively) (Fig. 2). Next, multivariate analyses revealed that nuclear GLI1 overexpression or FOXC2 expression could become independent prognostic factors for IBC patients in OS ($P=0.008$ and $P=0.007$, respectively) and in DFS ($P=0.024$ and $P=0.002$, respectively). Moreover, we also found that lymph node metastasis, HER-2 amplification and a basal-like subtype were each significant independent prognostic variables for DFS and OS.

Discussion

Sonic Hedgehog signaling controls a variety of developmental processes such as differentiation,

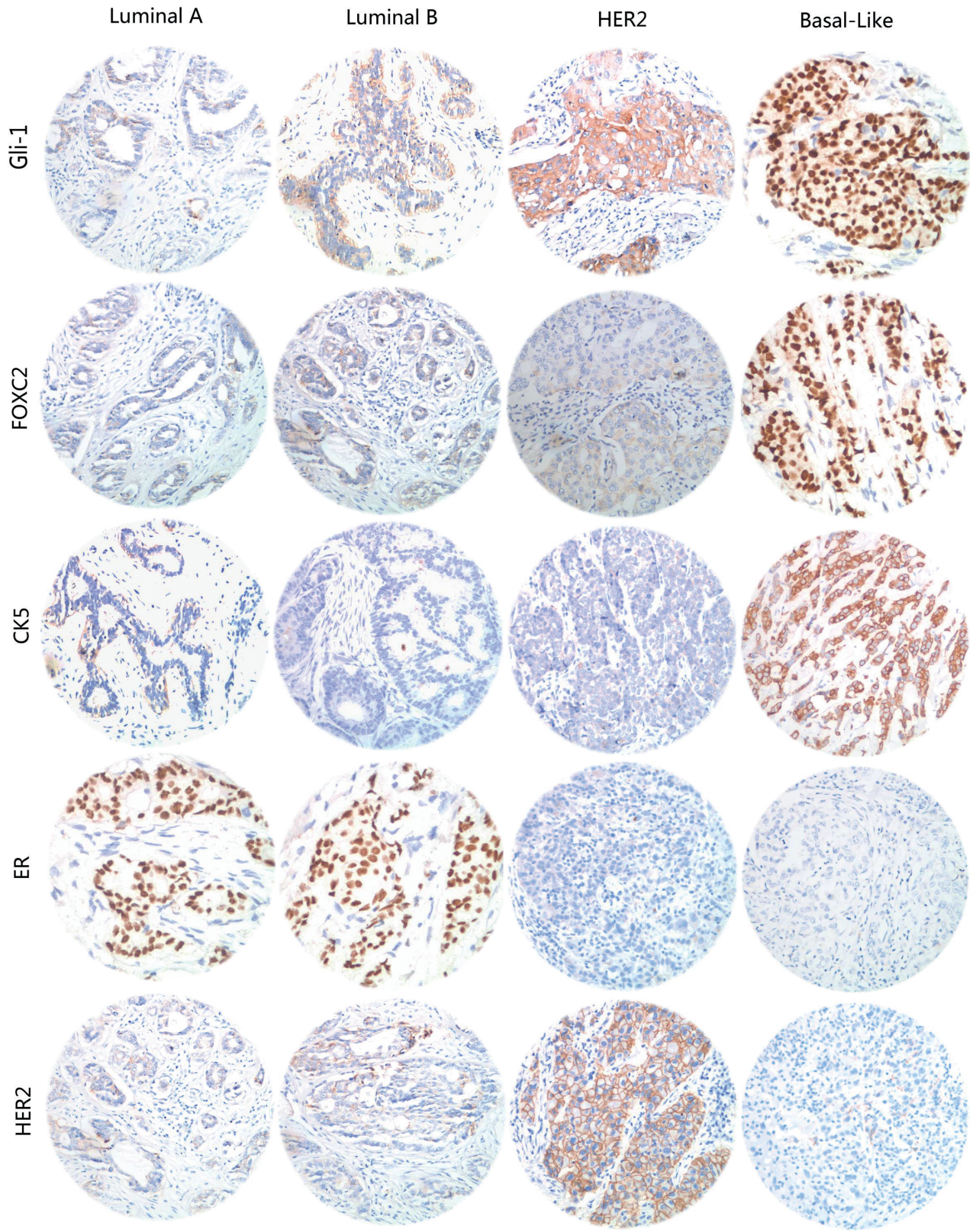

Fig. 1. Representative immunohistochemical staining of GLI1 and FOXC2 protein in the molecular subtype of invasive breast tumors, using a TMA.

Table 1. Clinicopathological and immunohistochemical parameters in relation to GLI1 and FOXC2 Immunoreactivity.

Variable		Total (n=262)	GLI1 expression						FOXC2 expression					
			Positive	%	P value	Nuclear overexpression	%	P value	Positive	%	P value	Nuclear overexpression	%	P value
Age	<56	138	116	84.1	0.904	56	40.6	0.314	101	73.2	0.636	30	21.7	0.447
	≥56	124	104	83.8		32	25.8		95	76.6		15	12.1	
Size-TNM	T1	93	75	80.6	0.715	30	32.3	0.504	74	79.6	0.211	12	12.9	0.307
	T2	115	96	83.5		36	31.3		88	76.5		20	17.4	
	T3	54	49	90.7		22	40.7		34	63.0		13	24.1	
Histological grade	Grade I	47	33	70.2	0.238	10	21.3	0.146	32	68.1	0.299	5	10.6	0.274
	Grade II	105	88	83.8		33	31.4		77	73.3		15	14.3	
	Grade III	110	99	90.0		45	40.9		87	79.1		25	22.7	
LV invasion	Present	139	126	90.6	0.085	58	41.7	0.113	113	81.3	0.137	29	20.9	0.328
	Absent	123	94	76.4		30	24.4		83	67.5		16	13.0	
LN metastasis	Positive	137	125	91.2	0.083	62	45.3	0.091	107	78.1	0.321	34	24.8	0.096
	Negative	125	95	76.0		26	20.8		89	71.2		11	8.8	
NPI	<3.4	90	71	78.9	0.425	22	24.4	0.117	59	65.6	0.212	11	12.2	0.091
	3.4-5.4	135	117	86.7		50	37.0		106	78.5		25	18.5	
	>5.4	37	32	86.5		16	43.2		31	83.8		9	24.3	
MIB-1	<10%	105	80	76.2	0.157	25	23.8	0.112	71	67.6	0.145	12	18.5	0.103
	10-30%	126	112	88.9		49	38.9		99	78.6		24	19.0	
	>30%	31	28	90.3		14	45.2		26	83.9		9	29.0	
ERα	Positive	187	151	80.7	0.087	47	25.1	0.031	132	70.6	0.072	19	10.1	0.019
	Negative	75	69	92.0		41	54.7		64	85.3		26	34.7	
PR	Positive	159	129	81.1	0.114	40	25.2	0.069	109	68.6	0.058	11	6.9	0.002
	Negative	103	91	88.3		48	46.6		87	84.5		34	33.0	
HER2 amplification	Positive	39	37	94.9	0.075	19	48.7	0.073	33	84.6	0.081	18	46.2	0.001
	Negative	223	183	82.1		69	30.9		163	73.1		27	12.1	
EGFR	Positive	37	32	86.5	0.457	19	51.4	0.077	70	81.1	0.247	14	37.8	0.084
	Negative	225	188	83.6		69	30.7		166	73.8		31	13.8	
P-cadherin	Positive	26	24	92.3	0.144	13	50.0	0.091	23	88.5	0.128	12	46.2	0.033
	Negative	236	196	83.1		75	31.8		173	73.3		33	14.0	
Cytokeratin 5/6	Positive	32	30	93.8	0.082	28	87.5	0.003	28	87.6	0.131	27	84.4	<0.001
	Negative	230	190	82.6		60	26.1		168	73.0		18	7.8	
Cytokeratin 14	Positive	30	27	90.0	0.079	26	86.7	0.004	29	96.7	0.004	23	76.7	<0.001
	Negative	232	193	83.2		62	26.7		167	72.0		22	9.5	
GLI1	Nuclear Positive	88										40	45.5	<0.001
	Nuclear Negative	174										5	2.9	
FOXC2	Nuclear Positive	45				40	88.9	<0.001						
	Nuclear Negative	217				48	22.1							
Molecular subtype	Luminal A	81	76	93.8	0.179	17	21.0	0.001	70	86.4	0.002	4	4.9	<0.001
	Luminal B	72	65	90.3		15	20.8		59	81.9		3	4.2	
	HER-2	41	20	48.8		11	26.8		16	39.0		6	14.6	
	Normal-likeBasa	35	26	74.3		13	37.1		19	54.3		2	5.7	
	Basal-like	33	33	100.0		32	97.0		32	97.0		30	90.0	

LV: lympho-vascular; LN: lymph node; NPI: Nottingham Prognostic Index; ERα: oestrogen receptor; PR: progesterone receptor; HER-2: human epidermal growth factor receptor 2; EGFR: epidermal growth factor receptor; GLI1: glioma-associated oncogene; FOXC2: factor forkhead-box C2.

proliferation, and organogenesis. Activation of the SHH/GLI cascade could promote tissue regeneration and repair in numerous organs. In counterbalance, it is believed that a deregulation of the SHH signaling network might lead to major tissue disorders and the development of a wide variety of aggressive and metastatic cancers (Mimeault and Batra, 2010). It is known that GLI1, an important member of the GLI family, is a vital positive activator of downstream target genes in the SHH/GLI signaling pathway. Nuclear localization of GLI1 protein is generally recognized as a hallmark defining its transcriptional activity (Kasper et al., 2006) . GLI1 consensus DNA-binding sequences identified in the 5'-regions of the cyclin D2 gene, suggest that GLI1 can bind directly with these downstream targets (Yoon et al., 2002) .

In addition, there are compact and intricate interactions between SHH signaling and the FOX

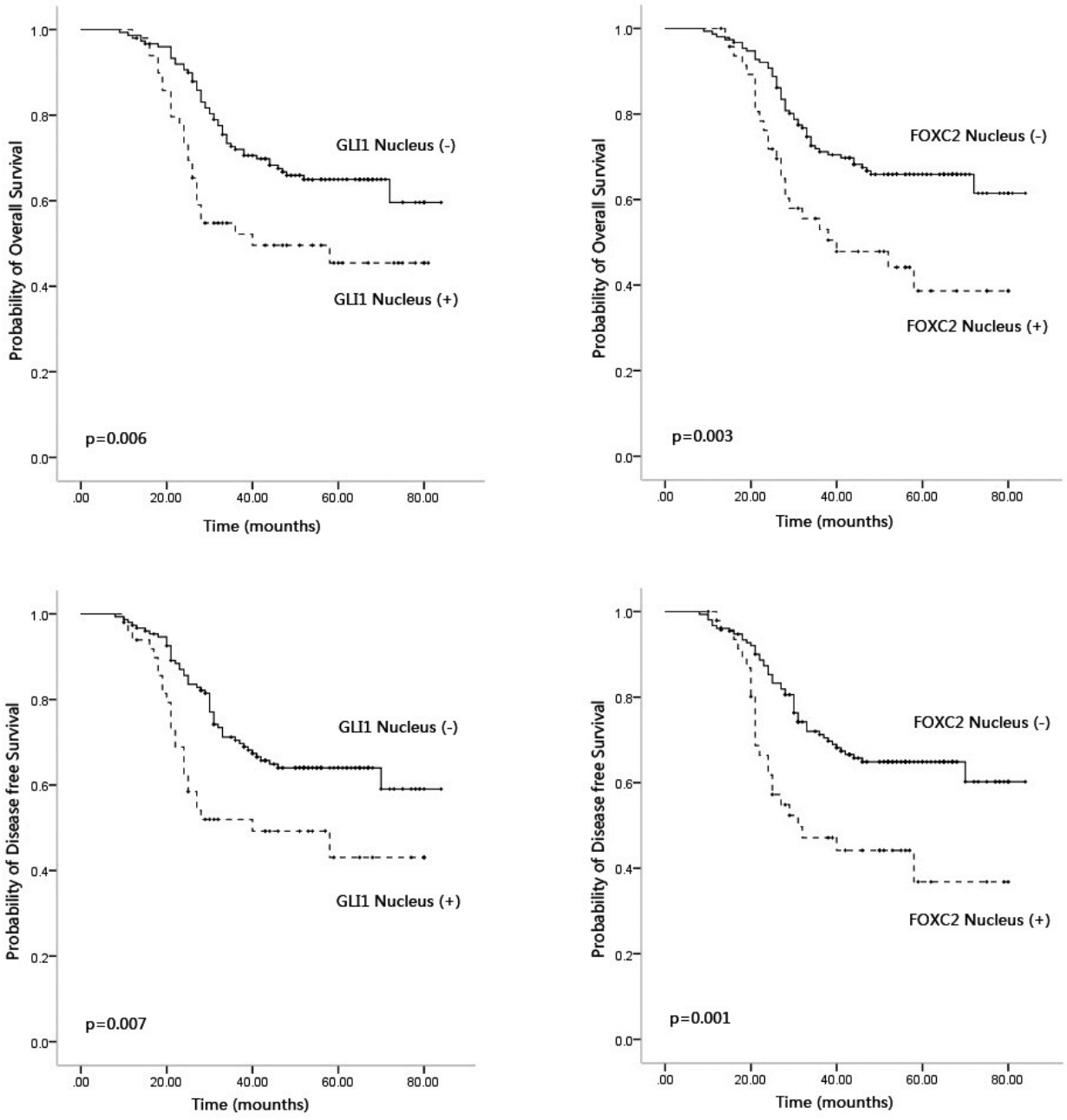

Fig. 2. Overall Survival (OS) and Disease-Free Survival (DFS) curves for breast cancer patients whose tumors were found to be nuclear GLI1 and FOXC2 positive vs. negative, as determined by immunohistochemical staining (GLI1: OS P=0.006, DFS P=0.007; FOXC2: OS P=0.003, DFS P=0.001).

Table 2. Multivariate analysis of disease-free survival (DFS) and overall survival (OS).

Variable	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
GLI1 Nucleus (Positive/Negative)	0.492	0.291-0.827	0.008	0.524	0.300-0.917	0.024
FOXC2 Nucleus (Positive/Negative)	0.711	0.419-0.884	0.007	0.425	0.249-0.732	0.002
HER2 amplification (Positive/Negative)	0.509	0.283-0.916	0.024	0.526	0.279-0.992	0.037
Histological grade I-II/III	0.727	0.379-1.395	0.024	0.929	0.467-1.848	0.834
LN metastasis (Positive/Negative)	0.233	0.093-0.585	0.002	0.207	0.082-0.522	0.001
Size-TNM T1-T2/T3	0.773	0.433-1.380	0.385	0.541	0.308-0.947	0.132
ERα (Positive/Negative)	0.393	0.219-0.706	0.086	0.508	0.288-0.895	0.065
Molecular subtype Basal-like/Others	0.241	0.115-0.504	<0.001	0.249	0.115-0.538	<0.001

transcription members, which regulate the development and maturation of some organs in human embryogenesis (Maeda et al., 2007). Recent analyses of GLI1 reveal potential involvement of some FOX protein family members in tumor transformation and developmental processes (Katoh et al., 2009). Indeed, Teh et al.(2002) found that SHH signaling up-regulated FOXM1 expression via GLI1, inducing its transcriptional activity in human basal cell carcinoma. The FOX protein member coded for by the transcription factor FOXC2 gene is also one of the SHH/GLI downstream target genes found to be involved in the development and progression of breast cancer, colonic adenocarcinoma and esophageal cancer (Myatt and Lam, 2007).

As GLI1 and FOXC2 are downstream target genes of SHH signaling, we sought to decipher whether there was a correlation between GLI1 and FOXC2 in breast cancer tissues. Our results showed that there is a highly significant correlation between the nuclear expression of these two SHH signaling members in invasive breast cancer cells, indicating that aberrant activation of GLI1 may lead to tumor proliferation, possibly modulated by FOXC2. The detailed interactions between GLI1 and FOXC2 will be further clarified in future research.

In a further analysis of the importance of the roles GLI1 and FOXC2 play in carcinogenesis of human breast cancer, we found that GLI1 protein expression was observed in 84% of breast cancers, while high nuclear immunoreactivity was observed in 33.6% of them. These were lower values than found earlier by Kubo et al. (2004) and Ten Haaf et al. (2009) in testing a smaller series of breast cancers, but the information is similar. In addition, we found that the expression of FOXC2 protein occurred in 75% of breast cancers, while nuclear immunoreactivity was observed in 17.1%. These data were slightly higher but in accordance with results from Mani et al. (2007), who found nuclear FOXC2 overexpression in 10% of human breast cancers. Although GLI1 and FOXC2 overexpression may not be a general characteristic of all human breast cancers, different laboratory results including our own do indicate that GLI1 and FOXC2 could be useful markers for human breast cancer. Nuclear localization of GLI1 is a valuable marker for evaluating the activation of the SHH pathway. These results strongly suggest that the SHH/GLI signaling pathway is quite extensively activated in breast cancers.

To assess the clinical significance and functional implications of GLI1 or FOXC2 in the development and progression of human breast cancer, we correlated GLI1 and FOXC2 expression data with clinicopathological characteristics such as age, tumor size, histological grade, lympho-vascular invasion, lymph node status, and amplification of NPI, ER, PR, HER-2 genes. We found that nuclear GLI1 or FOXC2 expression failed to have an association with these clinicopathological features, with the exception of there being an association with ER negativity. Although the correlation between GLI1 expression and clinicopathological characteristics in human breast cancer has been analyzed by several groups, their results are not yet entirely consistent. Kubo et al.(2004) found that there is a significant association of GLI1 overexpression with both histological type and with ER positivity. Ten Haaf et al. (2009) found a significant association of GLI1 overexpression with tumor stage and lymph node status. These associations could not be shown or corroborated by our results. Zhao et al. demonstrated that negative correlation between expression of ER and Gli1 in human breast cancer cell lines and GLI1 overexpression may be regulated through the down-regulation of both the expression and transactivation of ERα (Zhao et al., 2010). Our results also found that nuclear GLI1 expression has an association with ER negativity. We presumed GLI1 overexpression in breast cancer could be one of the mechanisms responsible for developing ER-independence. Additionally, our data demonstrating an association between FOXC2 and ER negativity indicated that FOXC2 overexpression may be regulated by the down-regulation of ERα, through an ER-independent mechanism

In carcinogenesis, cellular EMT resulting in invasive cancer must involve multiple genetic cellular changes that affect oncogenes or tumor transformation. It is likely that in IBC, the effect of GLI1 and FOXC2 proteins on cell differentiation and proliferation may reflect changes occuring within different genes and signaling pathways. As the activity seen was not universal in breast cancer, we began to assess whether GLI1 and FOXC2 play a greater role in the development of a few of the various subtypes of human breast cancer.

First, we compared the expression of nuclear GLI1

and FOXC2 within the various molecular subtypes. Interestingly, the associations between nuclear GLI1 overexpression or FOXC2 expression and some basal-like markers, namely CK5/6, CK14, EGFR and P-cadherin, which did suggest that GLI1 and FOXC2 could be important for the differentiation of malignant cells. We wondered if GLI1 and FOXC2, together with other differentiation-involved partners, might drive basal-like breast cancer cell differentiation. Although a role in growth and differentiation for GLI1 in normal mammary epithelial cells and in breast cancer tissue has already been described (Ten Haaf et al., 2009), a specific correlation of GLI1 expression with the basal-like breast cancers has not yet been reported. Moreover, our data for FOXC2 expression was in line with previously published works, which do state that FOXC2 is expressed in aggressive basal-like breast cancers, and is responsible for invasion and metastasis of breast cancers (Mani et al., 2007; Taube, 2010). As both GLI1 and FOXC2 are components of the SHH signaling pathway, we believe that finding a correlation of these two proteins with basal-like breast cancers could highlight the role that the SHH signaling pathway has in maintaining that basal-like differentiated phenotype. In addition, the study of their expression could improve our understanding of the relationship that GLI1 and FOXC2 may have in all breast cancer patients.

In our current prospective 5-year follow-up study, our data revealed that there was indeed a significant correlation between the overall survival rate of IBC patients and the presence and levels of nuclear GLI1 or FOXC2 expression. Although nuclear GLI1 or FOXC2 expression did not correlate solely with the important known prognosticators such as lymph node status and Her-2 amplification, the multivariate analyses that we performed suggested that nuclear GLI1 or FOXC2 expression, along with the nodal status, Her-2 amplification and basal-like subtype were jointly strong predictors of clinical outcome. Our finding is in line with work by Ten Haaf et al., who demonstrates that GLI1 expression alone can significantly predict a poor survival for breast cancer patients. Our data, obtained from a larger patient cohort, solidly extends the predictors to additional markers and pinpoints the breast cancer type they influence. In addition, Taube et al. (2010) found FOXC2 association with basal-like phenotype, but no survival association was reported. One possible reason for FOXC2 expression not being correlated with survival in Taube's study is that they used the cited microarray-based stratification of breast cancer patients, whereas we used immunohistochemistry staining to detect FOXC2 subcellular localization and correlated the nuclear expression of FOXC2 with the survival study. Another possible reason was that our data was obtained from a large patient cohort and should be more accurate. It has been know that FOXC1 and FOXC2 encoding closely related Fox transcription factors contain virtually identical DNA-binding domains (Kume et al., 2000). FOXC1 has been shown to be associated with poor prognosis and basal-like breast cancer (Ray et al. 2010, 2011). It is possible that FOXC2 antibody recognizes FOXC1 and play similar roles in the carcinogenesis of the breast cancer.

In conclusion, the results of our study suggest that there was not only a significant correlation between nuclear GLI1 and FOXC2 expression in human breast cancers, but that elevated levels of nuclear GLI1 or FOXC2 protein were specifically associated with the basal-like phenotype and that their presence conveyed a poor disease-free survival rate for breast cancer patients. These data indicated that the SHH signaling molecules GLI1 and FOXC2 may be involved in the differentiation, proliferation and invasion of tumor cells through the induction of a nuclear accumulation of GLI1 protein, followed by subsequent stimulation of the downstream target gene, FOXC2. These two proteins could be useful as specific molecular markers for diagnosis and as therapeutic targets for human basal-like breast cancers. Additional studies are warranted to better understand the biological significance of GLI1 and FOXC2 and eventually translate that information into clinically relevant solutions, in order to further optimize treatment of patients with basal-like breast cancer.

Authors' contributions: Yuan Li and Sheng Zhou carried out the immunohistochemistry studies, performed the statistical analyses and drafted the manuscript. Wentao Yang assisted with the experimental design and manuscript writing. Qin Yang participated in the immunohistochemistry studies. All authors read and approved the final manuscript.

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