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Original Contribution

Diagnostic and therapeutic ERβ, HER2, BRCA biomakers in the histological subtypes of lung adenocarcinoma according to the IASLC/ATS/ERS classification

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ABSTRACT

Several studies revealed that non–small cell lung cancers (NSCLCs) frequently express ER, PR, HER2 and carry BRCA mutation. However, these markers in histological subtypes of lung adenocarcinoma have not been thoroughly investigated. We retrospectively evaluated a total of 640 lung adenocarcinoma samples for ERα, ERβ, PR and HER2 expression by immunohistochemistry and western-blotting, for EGFR and BRCA mutation by real-time PCR and sequencing. Furthermore, HER2 amplification and mutation were explored in samples harboring immunopositivity HER2 using fluorescence in situ hybridization and real-time PCR, respectively. The micropapillary and invasive mucinous predominant adenocarcinoma were frequently detected the higher level of cytoplasmic ERβ (64.9% and 56.6%), HER2 (68.1% and 60.1%) protein expression. But, amplification of HER2 was detected in only three cases (3/110, 2.7%) and 26 HER2 mutations in 110 cases were identified (23.6%) in the HER2 immunopositivity patients. Logistic regression analysis showed that cytoplasmic ERβ ($P = 0.032$) and HER2 ($P = 0.015$) expression were independently associated with EGFR mutation. 8 patients (8/640, 1.25%) harbored pathogenic BRCA mutations, 6 with germline BRCA mutations and 2 with somatic BRCA1 mutations were detected with lacking ERβ, PR and HER2 expression. Acinar predominant adenocarcinoma had the higher percentage of BRCA mutations than other subtypes. A systematic examination of ERβ, HER2 and BRCA biomarkers could potentially be useful to diagnosis and identify patients with the histological subtypes of lung adenocarcinoma, who might benefit from the further individualized treatment of anti-hormone, anti-HER2 and/or PARP inhibitors therapeutics.

1. Introduction

Lung cancer is one of the most common cancers globally and is the leading cause of death in both females and males. Lung cancer in never-smokers is more frequently observed in women, according to geographical origin and patient selection. It suggests a possible involvement of gender-dependent factors in the pathogenesis and development of non-small cell lung cancer (NSCLC) [1]. Emerging evidence shows that female sex hormones, especially endogenous and exogenous estrogens, are key contributors to NSCLC progression in women [2].

Estrogen receptors (ERs) are members of the nuclear steroid receptor superfamily, and mediate cellular responses to the hormone estrogen. In human ERs, there are two isoforms (ERα and ERβ) with partial homology, yet distinct function, which are expressed with different tissue distributions or at various levels of the same tissue [2,3]. The previous studies reveal significant expression of estrogen receptors (ERα, ERβ) in both extranuclear and nuclear sites in most NSCLC [4]. Progesterone receptor (PR) is an estrogen response gene. There are several reports of expression of PR in NSCLC tissues, although there is a great deal of variability in the reported frequency of expression [4,5]. These hormone receptor markers may become useful biomarkers, potentially able to predict the aggressiveness of lung cancers and to identify patients who might respond to hormonal therapy.

The human epidermal growth factor receptor (HER) family is made up of four main members: HER-1, HER-2, HER-3, and HER-4. The HER2 (c-erbB-2 or neu), regulated by overexpression and/or gene amplification, has been proven important in many cancers, including breast and

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gastric cancer, in which overexpression of HER2 confers poor prognosis although it relates to possible benefit from specific anti-HER2 therapy [6]. Recent reports suggest correlations between ER-positive breast cancer and expression of HER2, whereas cross talk between ER and HER2 is reported to contribute to resistance to endocrine therapies [7]. Three principal mechanisms of HER2 alterations can be identified: HER2 protein overexpression, HER2 gene amplification and HER2 gene mutations. There are several methods for the detection of HER2 "positivity" in NSCLC, but no gold standard has been established. HER2 overexpression and amplification have been described in 6–35% and in 10–20%, respectively, of NSCLC patients [8]. HER2 mutations have been reported to exist in up to 4% of NSCLC and are more common in Asians, never smokers, women and adenocarcinomas [9]. The epidermal growth factor receptor (EGFR or HER-1 or erbB-1) is a transmembrane tyrosine kinase receptor, can dimerize and mediate a series of signaling cascades via phosphorylations that culminate in the regulation of transcription factors and other proteins that control cell fate [10]. EGFR is mutated or overexpressed in many human cancers, including NSCLC tumors. This fact has been determinant for the approval of the EGFR receptor kinase inhibitor for clinical use in the treatment of advanced NSCLC as monotherapy following failure of chemotherapy [11]. Furthermore, the crosstalk between EGFR and ER pathways in lung cancer could provide a rationale to combine inhibitors of EGFR activation with anti-estrogen therapy for the treatment of the disease [12].

Breast cancer susceptibility type 1 and 2 genes (BRCA1 and BRCA2) are tumor suppressor genes that participate in the DNA repair processes through homologous recombination repair (HRR) to the maintenance of genomic integrity. Approximately, 5% of breast cancer cases and 20%–30% of ovarian cancers are caused by germline alterations in the BRCA1 and BRCA2 genes [13]. Previous results showed that gBRCAm additionally conferred increased susceptibility to a spectrum of other cancers, including lung [14]. Furthermore, clinical trials have demonstrated an excellent response rate of gBRCAm carriers to PARP inhibitors [15]. Association between the triple-negative phenotype, lacking ER, PR, and HER2 protein expression, and breast cancers harboring germline mutations in the BRCA gene has been well-described [16]. Like breast cancer, lung adenocarcinoma may be a hormone and BRCA related carcinoma.

A new lung adenocarcinoma classification that is based on predominant histologic patterns was proposed by the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS) in 2011 [17]. This classification clearly emphasizes the prognostic significance of histologic subtypes, which have been validated in independent cohorts [18]. Several previous studies have identified the significant associations of the driver gene mutations and the predominant subtypes according to the proposed IASLC/ATS/ERS classification [19,20]. Recently, the positive association between ER expression and EGFR mutations has been detected [11], and the potential clinical impact of ERα, ERβ, and PR has also been investigated in lung cancers [3,21,22]. Despite these investigations, the associations between the new classification of histologic subtypes and ER, PR, HER2 expression in lung adenocarcinoma have not been thoroughly investigated. Meanwhile, to date, the prevalence of BRCA in histologic subtypes of Chinese lung adenocarcinoma remains elusive.

In our study, we investigate whether ER, PR and HER2 expression have any associations with clinicopathologic factors, histologic patterns, including EGFR mutation status, in 640 lung adenocarcinoma patients. Then, we examined the association between BRCA1/2 mutations and lung adenocarcinoma. These findings may provide a certain basis for further individualized treatment of the histological subtypes of lung adenocarcinoma, according to the IASLC/ATS/ERS classification.

2. Materials and methods

2.1. Patients and tissue specimens

A total of 640 never-smoking lung adenocarcinoma cases met eligibility for this study. The specimens were obtained from patients who underwent complete surgical resection from 2014 to 2019 at the First Affiliated Hospital of Dalian Medical University. The clinicopathological factors of the patients are shown in Table 1. Tumors were classified according to the IASLC/ATS/ERS classification system by means of comprehensive histologic subtyping, with the percentage of each histologic component recorded in 5% increments [17]. The stage was determined according to the 8th edition of the TNM classification for lung and pleural tumors. All of the procedures were approved by the Ethics Committee on Human Research of the First Affiliated Hospital of Dalian Medical University, and written informed consent for the use of their tissue and/or blood samples was obtained from all of the patients before surgery.

2.2. Immunohistochemical analysis

Serial tissue sections of 4-μm thickness sliced from paraffin-embedded specimens were used for immunohistochemistry using the labeled streptavidin-biotin method. Immunostaining was performed with the antibodies, ERα (F-10, 1:100, Santa Cruz Biotechnology, USA), ERβ (B-1, 1:100, Santa Cruz Biotechnology, USA), PR (1E2, Ventana, Tucson, AZ, USA) and HER2 (4B5, Ventana, Tucson, AZ, USA). The slides were deparaffinized with xylene and rehydrated with ethanol. For ERα and ERβ, antigen retrieval was carried out by autoclaving the slides in citrate buffer (0.01 mol/L) at 100 °C for 3 min. For PR and HER2, immunohistochemical staining was performed using BenchMark XT (Ventana, Tucson, AZ, USA), an automatic immunohistochemical staining system. Positive immunoreactivity for ERα, ERβ, and PR were counted among 1000 cells per case and was recorded as "positive" for positive results of more than 10% [23]. ERα, ERβ and PR expression were evaluated separately in tumor nuclei and cytoplasm. The scoring method for HER2 expression is based on the cell membrane staining. (1) 3+: positive HER2 expression, uniform intense complete membrane strong staining of more than 10% of invasive tumor cells; (2) 2+: nonuniform complete membrane weak staining >10% or uniform intense complete membrane strong staining <10% of invasive tumor cells; (3) 1+: incomplete membrane weak staining in at least 10% of cells; (4) 0: negative for HER2 protein expression [24]. All available tumor slides were reviewed by two pathologists, using an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a standard 22-mm diameter eyepiece.

2.3. Nuclear/cytoplasmic protein extraction and Western blotting

Nuclear and cytoplasmic extraction (Pierce Biotechnology, Inc., USA) was performed according to the manufacturer's instructions. For western-blotting in formalin-fixed, paraffin-embedded tissue blocks (FFPE), total cell proteins were resolved by 7.5% SDS-PAGE, transferred to polyvinylidene difluoride membranes (90 V for 2 h), and probed with monoclonal antibodies, using ERβ(B-1) 1:400, lamin B1(B-10) 1:500, tubulin(5F131) 1:200, β-actin(C4) 1:500 and HER2(F-11) 1:300 (Santa Cruz Biotechnology, USA). Bound antibody was detected by incubation with HRP conjugated secondary antibody (1:5000, Zhongshan Golden Bridge, China) and processed using enhanced chemiluminescence.

2.4. Gene mutation analysis

Genomic DNA was extracted from a 5-mm cube of tumor tissue using a MagPure FFPE DNA LQ Kit (Magen Biotech Co., Ltd., Germany) and subsequently diluted to 2 ng/μL. EGFR 18–21 exon mutation and HER2 exon 20 mutation between codons 775 and 881 were detected using a

Table 1
Clinicopathological characteristics of ERα, ERß, PR, HER2 expression and EGFR, BRCA mutation status in 640 never-smoking lung adenocarcinoma patients.

Parameter	ERα(N)+/total (%)	ERα(C)+/total (%)	ERß(N)+/total (%)	ERß(C)+/total (%)	PR(N)+/total (%)	HER2+/total (%)	EGFR+/total (%)
Gender							
Male	42/246(17.1)	7/246(2.8)	127/246(51.6)	112/246(45.5)	20/246(8.1)[b]	72/246(29.3)	79/246(32.1)[a]
Female	90/394(22.8)	12/394(3.0)	192/394(48.7)	177/394(44.9)	72/394(18.3)	113/394(28.7)	161/394(40.9)
Age							
<60	45/310(13.9)[b]	13/310(4.2)	109/310(35.2)[b]	76/310(24.5)[b]	41/310(13.2)	108/310(34.8)[a]	138/310(44.5)[b]
≥60	87/330(26.4)	6/330(1.8)	210/330(63.6)	213/330(64.5)	51/330(15.5)	77/330(23.3)	102/330(30.9)
Menopause							
Premenopausal	72/180(40.0)[b]	10/180(5.6)[a]	109/180(60.6)[b]	124/180(68.9)[b]	61/180(33.9)[b]	118/180(65.6)[b]	64/180(35.6)[a]
Postmenopausal	18/214(8.4)	2/214(0.9)	83/214(38.8)	53/214(24.8)	11/214(5.1)	67/214(31.3)	97/214(45.3)
Pathologic stage							
IA	34/131(26.0)[b]	4/131(3.1)	87/131(66.4)[b]	62/131(47.3)	12/131(9.2)	18/131(13.8)[b]	28/131(21.4)[b]
IB	39/170(22.9)	6/170(3.5)	67/170(39.4)	79/170(46.5)	23/170(13.5)	14/170(8.2)	37/170(21.8)
IIA	17/124(13.7)	2/124(1.6)	55/124(44.4)	52/124(41.9)	18/124(14.5)	47/124(37.9)	67/124(54.0)
IIB	16/136(11.8)	5/136(3.7)	62/136(45.6)	53/136(39.0)	24/136(17.6)	56/136(14.2)	71/136(52.2)
IIIA	12/45(26.7)	2/45(4.4)	31/45(68.9)	25/45(55.6)	9/45(20.0)	37/45(82.2)	24/45(53.3)
IIIB	14/28(50.0)	0/28(0.0)	16/28(57.1)	16/28(57.1)	6/28(21.4)	12/28(42.9)	11/28(39.3)
IV	0/6(0.0)	0/6(0.0)	1/6(16.7)	2/6(33.3)	0/6(0.0)	1/6(16.7)	2/36(33.3)
Pleural invasion							
Absent	104/538(19.3)	14/538(2.6)	305/538(56.7)[b]	213/538(39.6)[b]	87/538(16.2)[a]	132/538(24.5)[b]	194/538(36.1)
Present	28/102(27.5)	5/102(4.9)	14/102(13.7)	76/102(74.5)	5/102(4.9)	53/102(52.0)	46/102(45.1)
Lymphatic invasion							
Absent	107/574(18.6)[b]	17/574(3.0)	309/574(53.8)[b]	247/574(43.0)[a]	89/574(15.5)[a]	165/574(28.7)	218/574(38.0)
Present	25/66(37.9)	2/66(3.0)	10/66(15.2)	42/66(63.6)	3/66(4.5)	20/66(30.3)	22/66(33.3)
Vascular invasion							
Absent	123/604(20.3)	18/604(3.0)	315/604(52.2)[b]	283/604(46.9)[b]	91/604(15.1)[a]	180/604(29.8)	228/604(37.7)
Present	9/36(25.0)	1/36(2.8)	4/36(11.1)	6/36(16.7)	1/36(2.8)	5/36(13.9)	12/36(33.3)
Predominant subtype							
AIS	8/60(13.3)[b]	0/60(0)[a]	47/60(78.3)[b]	10/60(16.7)[b]	4/60(6.7)[b]	0/60(0)[b]	6/60(10.0)[b]
MIA	16/74(21.6)	0/74(0)	56/74(75.7)	13/74(17.6)	8/74(10.8)	6/74(8.1)	15/74(20.3)
Lepidic	16/76(21.1)	5/76(6.6)	60/76(78.9)	11/76(14.5)	16/76(21.1)	14/76(18.4)	40/76(52.6)
Acinar	40/94(42.6)	8/94(8.5)	40/94(42.6)	47/94(50.0)	28/94(29.8)	21/94(22.3)	60/94(63.8)
Papillary	36/82(43.9)	2/82(2.4)	39/82(47.6)	40/82(48.8)	20/82(24.4)	20/82(24.4)	49/82(59.8)
Micropapillary	8/94(8.5)	1/94(1.1)	30/94(31.9)	61/94(64.9)	8/94(8.5)	64/94(68.1)	20/94(21.3)
Solid	8/72(11.1)	1/72(1.4)	14/72(19.4)	56/72(77.8)	8/72(11.1)	14/72(19.4)	36/72(50.0)
IMA	0/76(0)	2/76(2.6)	29/76(38.2)	43/76(56.6)	0/76(0)	46/76(60.5)	14/76(18.4)
Enteric	0/12(0)	0/12(0)	4/12(33.3)	8/12(66.7)	1/12(8.3)	2/12(16.7)	0/12(0)
Total							
Negative	508/640(79.4)	621/640(97.0)	321/640(50.2)	351/640(54.8)	548/640(85.6)	455/640(71.1)	400/640(62.5)
Positive	132/640(20.6)	19/640(3.0)	319/640(49.8)	289/640(45.2)	92/640(14.4)	185/640(28.9)	240/640(37.5)
Pathogenic mutation							
gBRCA1m	0/4(0)	0/4(0)	2/4(50.0)	0/4(0)	0/4(0)	0/4(0)	1/4(25.0)
gBRCA2m	0/2(0)	0/2(0)	0/2(0)	0/2(0)	0/2(0)	0/2(0)	1/2(50.0)
sBRCA1m	0/2(0)	0/2(0)	0/2(0)	0/2(0)	0/2(0)	0/2(0)	0/2(0)
sBRCA2m	0/0(0)	0/0(0)	0/0(0)	0/0(0)	0/0(0)	0/0(0)	0/0(0)

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; The predominant subtype according the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; IMA, invasive mucinous adenocarcinoma; gBRCAm, germline BRCA mutation; sBRCAm, somatic BRCA mutation; N, Nuclear; C, Cytoplasmic.

[a] Significantly correlated (χ^2 test, $P < 0.05$).

[b] Significantly correlated (χ^2 test, $P < 0.001$).

Amplification Refractory Mutation System (ARMS) and Polymerase Chain Reaction (PCR) according to the manufacturer's instructions (AmoyDx, Ltd., Xiamen, China), followed by an ABI 7500 Real-time PCR Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., USA).

2.5. Fluorescence in situ hybridization (FISH)

HER2 FISH were performed and interpreted according to manufacturers' test kit protocols (PathVysion Kit, USA). The assay is performed on unstained 5 μm-thick sections from FFPE tissue blocks. Scoring is performed by two pathologists. Two-colour FISH analysis was performed by counting chromosome 17 centromere signals (CEP17; green) and HER2 gene-specific signals (red) within 20 nuclei of invasive cancer cells and then calculating the ratio of red:green signals. (1) positive HER2 amplification: FISH ratio ≥ 2 or ratio < 2 and but the Average HER2 Signal Number ≥ 6.0 or HER2 signals are connected as cluster; (2) equivocal HER2 amplification: FISH Ratio < 2.0 and the Average HER2 Signals Number between 4.0– 6.0, the specimen is undetermined, that require to count additional 20 cells and recalculate to interpret the result; (3) negative HER2 amplification: FISH Ratio < 2.0 and the Average HER2 Signals Number < 4.0. (4) >3 CEP17 copies per cell were noted as polysomy [23].

2.6. Next generation sequencing (NGS)

Next generation sequencing was performed using FFPE-isolated tumor DNA with a total input of 500–1000 ng per sample. The mean tumor cell percentage of the included samples was 76% (range: 50%–95%). NGS was performed using the commercially available ADx BRCA1/2 Analysis Kits (AmoyDx, Ltd., Xiamen, China). The libraries were prepared using SureSelectQXT Library Prep Kit (Agilent) according to manufacturer's instructions and sequenced on Nextseq 500/Miseq (Illumina, San Diego, CA). A minimum of 50 ng of DNA is required for NGS library construction. For each patient, 2 μL of genomic DNA (25 ng/μL) was used for enzymatic fragmentation. Library amplification was performed using Herculase II Fusion DNA Polymerase (Agilent) and the PCR product was purified using the Agencourt AMPureXP purification bead system (Beckman Coulter; Pasadena, CA). The targeted DNA was

captured using streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin T1; Thermo Fisher Scientific, Waltham, MA) followed by indexing (SureSelectQXT P7 and P5 dual indexing primers). The analysis of amplified indexed library DNA was performed using High Sensitivity D1000 ScreenTape (on Agilent TapeStation). Two samples were excluded (DNA quantity and quality) and 31 were multiplexed into 1.4 pM pool and loaded onto the Nextseq 500/Miseq. Pathogenic mutations were determined by a clinical molecular geneticist according to the guidelines of American College of Medical Genetics (ACMG); class 1 = benign, class 2 = likely benign, class 3 = variant of unknown significance (VUS), class 4 = likely pathogenic, and class 5 = pathogenic [25].

2.7. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 22.0 (IBM Co., Armonk, NY, USA). Pearson's χ^2 test (when no cell of a contingency table has expected count less than five) or Fisher's exact test (when any cell of a contingency table has expected count less than five) was used to assess the association between two categorical variables. Independent sample *t*-test was applied to investigate correlation between a categorical variable and a continuous variable. For multivariate analyses, logistic regression model was employed. *P*-values were two-tailed for all the tests. Statistical significance $P < 0.05$ was deemed to indicate statistical significance.

3. Results

3.1. Expression of receptors for steroid hormone in lung adenocarcinoma tissues

A total of 640 never-smoking lung adenocarcinoma cases, all patients were Chinese, were performed to evaluate the expression of steroid hormones, ER and PR by immunohistochemistry. Specific subcellular staining, at nuclear and cytoplasmic level of ERα, ERβ and PR was independently recorded. We found that about 3.0% (19/640) and 45.2% (280/640) of samples expressed ERα and ERβ at cytoplasmic level, while 20.6% (132/640) and 49.8% (319/640) of them expressed nuclear ERα and ERβ respectively (Table 1). Our study revealed significant expression of the nuclear ERα and both extranuclear and nuclear ERβ in lung adenocarcinoma samples. A higher percentage of samples showed staining for ERβ than ERα. We also reported further on the expression of PR protein, we found that only 14.4% (92/640) of lung adenocarcinoma samples showed nuclear staining (Table 1), while PR expression at cytoplasmic level was not found in any sample.

As staining ERβ was predominant ER isoform in tissue samples of lung adenocarcinoma, western blotting was used to evaluate subcellular localization of ERβ in immunopositivity tissues of the micropapillary predominant subtype ($n = 20$ cases) and the invasive mucinous predominant subtype ($n = 20$ cases) lung adenocarcinoma, paired non-tumorous lung tissues (>5 cm far from the tumorous tissues) of the same case. Non-tumorous counterparts almost only showed the expressed nuclear ERβ and little cytoplasmic ERβ, while the significant higher level of cytoplasmic ERβ was found in lung adenocarcinoma samples in comparison to the non-tumorous counterparts (Fig. 1A, $P <$

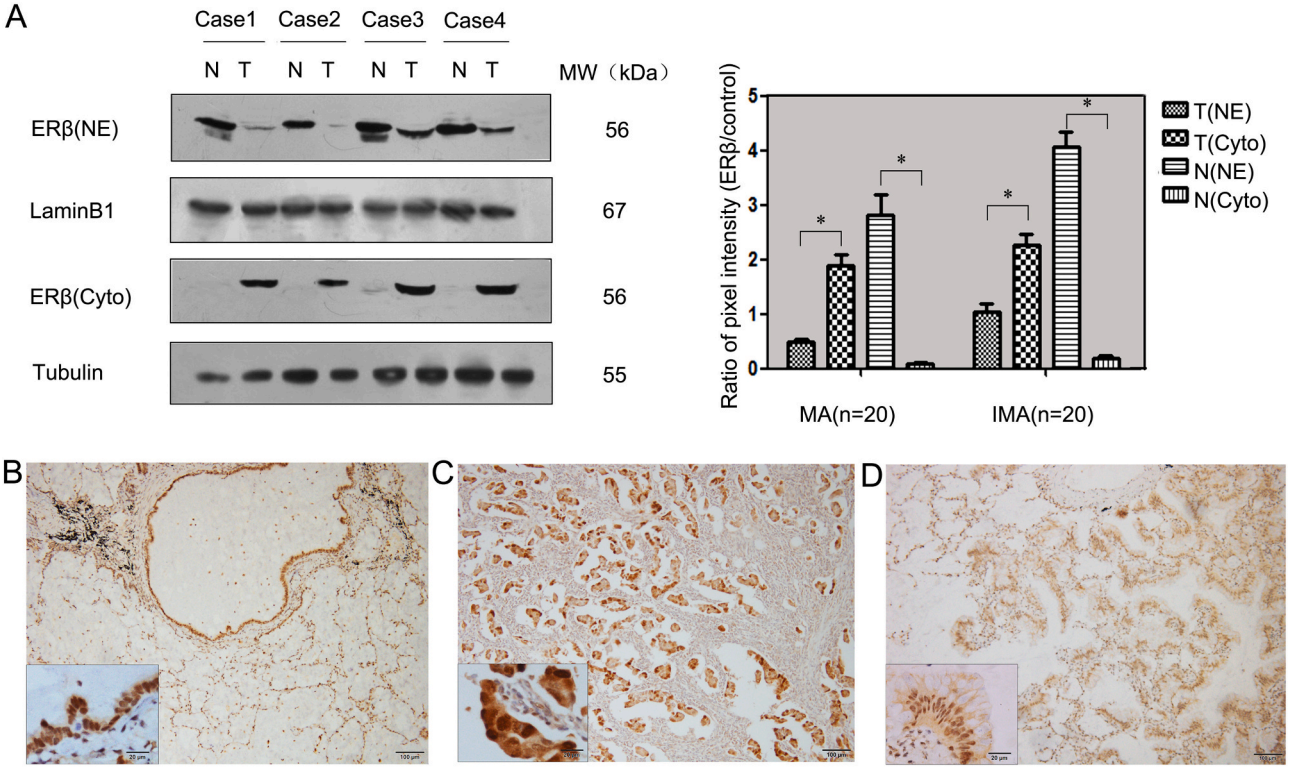

Fig. 1. Representative expression of ERβ in lung adenocarcinoma.
(A) Matched tumourous (T) and surrounding non-tumorous (N) tissues from 20 micropapillary predominant subtype adenocarcinoma (MA) and 20 invasive mucinous predominant subtype adenocarcinoma (IMA) patients were analyzed by western blotting. The efficiency of cytoplasmic (Cyto) and nuclear (NE) fraction was assessed by tubulin and lamin B1, respectively. For the quantification of band density, the fold elevation of ERβ in the cytoplasm versus the nucleus was displayed as the mean ± SD. Columns, mean ($n = 20$), *$P < 0.05$. (B) Normal bronchial epithelium, positive staining of nuclear ERβ by immunohistochemical staining. Scale bars represent 100 μm and 20 μm. (C) Micropapillary predominant subtype adenocarcinoma, positive staining of nuclear and cytoplasmic ERβ by immunohistochemical staining. Scale bars represent 100 μm and 20 μm. (D) Invasive mucinous predominant subtype adenocarcinoma, positive staining of nuclear and cytoplasmic ERβ by immunohistochemical staining. Scale bars represent 100 μm and 20 μm.

0.05). These data suggest that the subcellular redistribution of ERβ may exist in tumorous and non-tumorous lung adenocarcinoma samples.

3.2. Associations between receptors for steroid hormone and histological subtypes in lung adenocarcinoma tissues

According to the IASLC/ATS/ERS classification, we analyzed receptors for steroid hormone (ERα, ERβ and PR) in predominated histologic subtypes of the lung adenocarcinoma tissue samples. Only nuclear positive ERβ was observed in normal bronchial epithelium (Fig. 1B). As shown in Table 1, compared with the other subtypes, nuclear ERβ were more common in patients with the adenocarcinoma in situ subtype (47/60, 78.3%; $P < 0.001$) and the lepidic predominant subtype (60/76, 78.9%; $P < 0.001$). While the micropapillary predominant subtype (61/94, 64.9%; $P < 0.001$) and the invasive mucinous predominant subtype (43/76, 56.6%; $P < 0.001$) were more frequently detected in cytoplasmic ERβ compared with other subtypes (Fig. 1C,D). Expressed nuclear ERα were more common in patients with the acinar predominant subtype (40/94, 42.6%; $P < 0.05$) and the papillary predominant subtype (36/82, 43.9%; $P < 0.05$). Regarding of PR, it occurred most frequently in the acinar predominant subtype (28/94, 29.8%; $P < 0.001$) and the papillary predominant subtype (20/82, 24.4%; $P < 0.001$), and less frequently occurred in the other subtypes.

As the micropapillary and invasive mucinous predominant subtype also have a poor prognosis similar to adenocarcinomas with a predominant solid subtype [17], indicated that the level of cytoplasmic ERβ was associated with bad prognosis in the lung adenocarcinoma.

3.3. HER2 in lung adenocarcinoma tissues, according to the IASLC/ATS/ERS classification

HER2 gene, regulated by overexpression and/or gene amplification, has been proven important in many cancers. The expression of HER2 was 28.9% (185/640) in our study (Table 1). The negative staining of HER2 was detected in normal bronchial epithelium (Fig. 2A). However, the statistic analysis indicated that HER2 was found more frequently in the micropapillary predominant subtype (64/94, 68.1%; $P < 0.001$) and the invasive mucinous predominant subtype (46/76, 60.5%; $P < 0.001$) than the other subtype group (Table 1, Fig. 2B,C). Lung adenocarcinoma is a heterogeneous tumor that includes various histological subtypes (Fig. 2D). In our study, one lung adenocarcinoma sample was found expressed HER2 in the micropapillary subtype and expressed ERβ in the acinar subtype (Fig. 2E,F). It showed that the HER2 and ERβ were expressed exclusively and characterized by distinct histological subtypes, molecular alterations.

Then, western blotting was used to evaluate in immunopositivity lung adenocarcinoma of the micropapillary predominant subtype ($n = 20$ cases) and the invasive mucinous predominant subtype ($n = 20$ cases), paired non-tumorous lung tissues of the same case. The significant higher level of HER2 expression was found in lung adenocarcinoma samples in comparison to the non-tumorous counterparts (Fig. 2G, $P < 0.05$).

An additional analysis was performed to assess for HER2 gene copy number alterations by FISH in 64 positive HER2 for the micropapillary predominant subtype and 46 positive HER2 for the invasive mucinous predominant subtype (Table 2). Amplification of HER2 was detected in only three cases of the invasive mucinous predominant subtype. FISH analysis was documented presence of low polysomy in some cases. None of the micropapillary predominant subtypes were positive for HER2 amplification (Fig. 2H,I,J).

Of 110 lung adenocarcinoma samples that resulted almost negative for FISH tested previously, we used real-time PCR to test the HER2 mutations, included in-frame insertions in exon 20 (from 3 to 12 bp) between codons 775 and 881, of which were the 12-bp duplication/insertion of YVMA at codon 775 and point mutations, L775S and G776C. 26 HER2 mutations in 110 cases were identified (23.6%), all mutually exclusive with EGFR mutations in 18–21 exon. We next analyzed whether there was any correlation with statistical significance between the HER2 levels of IHC-positive expression and HER2 mutation or amplification. However, there was no association (Table 2, $P > 0.05$). No HER2 mutations were detected among HER2 amplification. Therefore, HER2 mutation was not associated with concurrent HER2 amplification.

3.4. Association between expression of ER, PR, HER2 and clinicopathologic features in lung adenocarcinoma

Possible relationships between steroid hormone receptors (ERα, ERβ and PR), HER2 immunostainings and clinicopathological features relevant in the lung adenocarcinoma patients were summarized in Table 1. We found that nuclear immunostaining for ERα was associated with age, menopause, pathological stage, lymphatic invasion ($P < 0.001$). Nuclear ERβ was associated with age, menopause, pathological stage, pleural invasion, lymphatic invasion, vascular invasion ($P < 0.001$). Cytoplasmic ERβ expression was the same as nuclear ERβ, except no association with pathological stage ($P > 0.05$). However, PR status was associated with gender, menopause ($P < 0.001$); pleural invasion, lymphatic invasion, vascular invasion ($P < 0.05$). Besides, PR was the only one steroid hormone receptor statistically significant association with gender ($P < 0.001$). Regarding of HER2 expression, it was associated with patient age ($P < 0.05$) and menopause, pathological stage, pleural invasion ($P < 0.001$).

3.5. Association between EGFR mutation and clinicopathologic factors in lung adenocarcinoma

The tumors of 240 (37.5%) patients were identified as EGFR mutant (EGFR-mut) and those of 400 (62.5%) patients were identified as EGFR wild-type (EGFR-wt) (Table 1). The percentage of EGFR-mut group was significantly higher in acinar-predominant invasive adenocarcinomas, compared with the other subtypes (63.8%, vs. 33.0%, respectively, Table 1). We next analyzed the correlation with statistical significance between the EGFR mutation and clinicopathological features (Table 1). We found that EGFR mutation was associated with gender ($P < 0.05$), age ($P < 0.001$), menopause ($P < 0.05$) and pathological stage ($P < 0.001$). Then, correlations among EGFR mutations with clinicopathological features were further evaluated by logistic regression analysis incorporating ERα, ERβ, PR, HER2 and histological subtypes. We used median age (60 years) as cutoff value, 95% confidence intervals (CI), and *P*-values were listed in Table 3. In the multivariate analysis, mutations in the EGFR gene were significantly associated with gender ($P = 0.035$), histology subtypes ($P = 0.018$) and expression of cytoplasmic ER-β ($P = 0.032$), HER2 ($P = 0.015$). However, the logistic regression indicated that this association of pathological stage disappeared when other factors were included. We found a significant, association between EGFR-mut and cytoplasmic ERβ expression ($P = 0.032$). On the contrary, there was no association between the expression of ERβ ($P = 0.548$) and ERα ($P = 0.446$) at nuclear level. The EGFR-mut did not correlate with expression of PR ($P = 0.885$). The above findings indicated that there was a close correlation between the expression of cytoplasmic ERβ, HER2 and the presence of EGFR mutations.

3.6. BRCA mutation in lung adenocarcinoma according to the IASLC/ATS/ERS classification

In addition to analyzing the EGFR mutation status of the lung cancer patients in our cohort, we investigated somatic and germline BRCA mutations. Of the 640 NSCLC patients screened, we detected 8 patients (8/640, 1.25%) harbored pathogenic or likely pathogenic BRCA mutations, 6 with germline BRCA mutation (4 gBRCA1m and 2 gBRCA2m) and 2 with somatic BRCA1 mutation (Tables 1, 4). Among the pathogenic gBRCAm and sBRCAm variants, we observed three types of mutations, with frameshift mutations being the most predominant mutation

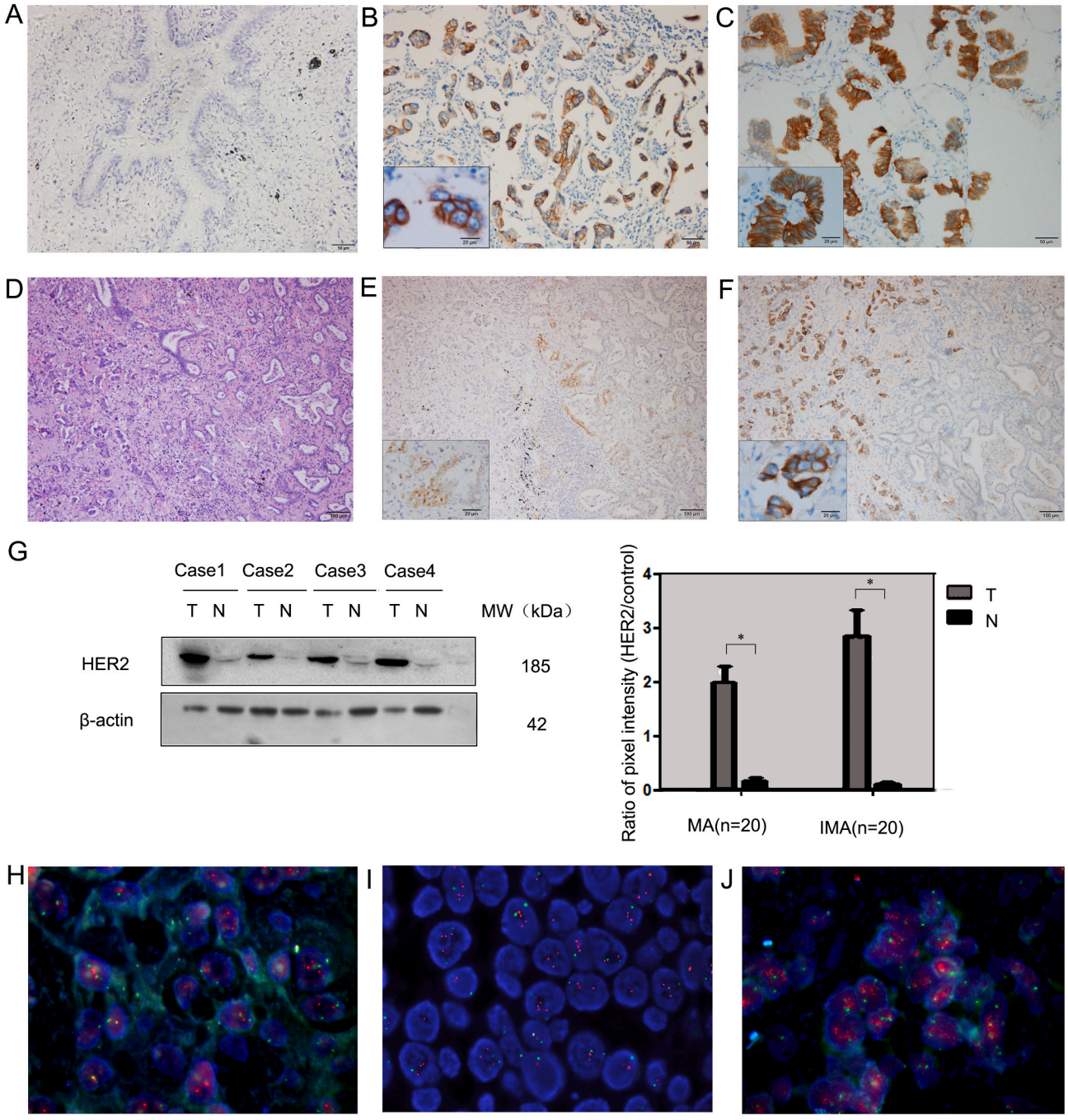

Fig. 2. Representative expression of HER2 in lung adenocarcinoma.
(A) Normal bronchial epithelium, negative staining of HER2 by immunohistochemical staining. Scale bar represents 50 μm. (B) Micropapillary predominant subtype adenocarcinoma, positive staining of HER2 by immunohistochemical staining. Scale bars represent 50 μm and 20 μm. (C) Invasive mucinous predominant subtype adenocarcinoma, positive staining of HER2 by immunohistochemical staining. Scale bars represent 50 μm and 20 μm. (D) Heterogeneous acinar and micropapillary predominant subtype lung adenocarcinoma, HE staining. Scale bar represents 100 μm. (E) Positive staining of ERβ in acinar predominant subtype compartment by immunohistochemical staining. Scale bars represent 100 μm and 20 μm. (F) Positive staining of HER2 in micropapillary predominant subtype compartment by immunohistochemical staining. Scale bars represent 100 μm and 20 μm. (G) Matched tumourous (T) and surrounding non-tumorous (N) tissues from 20 micropapillary predominant subtype adenocarcinoma (MA) and 20 invasive mucinous predominant subtype adenocarcinoma (IMA) patients were analyzed by western blotting. The ratio between the density of HER2 and β-actin of the same patient was calculated and expressed graphically. Data are expressed as the means ± SD. Columns, mean ($n = 20$), *$P < 0.05$. (H) Dual color FISH analysis was performed using chromosome 17 centromere signals (CEP17; green) and HER2 gene-specific signals (red). FISH-negative, non-polysomy, ×1000 magnification. (I) Low polysomy of HER2, with>3 copies of HER2 in 20% of nuclei counted by FISH, ×1000 magnification. (J) FISH-positive, HER2 amplification showing a HER2 gene/chromosome 17 ratio >2 as cluster in 80% cells by FISH, ×1000 magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Amplification and mutation of HER2 according to their positive immunostaining in the micropapillary and invasive mucinous predominant lung adenocarcinoma.

Immunostaining	Total	FISH amplification/total (%)		*P*-value	HER2 mutation/total (%)		*P*-value
		+	−		+	−	
Micropapillary predominant	64						
HER2 (1+)	12	0/12(0.0)	12/12(100)		1/12(8.3)	11/12(91.7)	0.284
HER2 (2+)	29	0/29(0.0)	29/29(100)		9/29(31.0)	20/29(69.0)	
HER2 (3+)	23	0/23(0.0)	23/23(100)		7/23(30.4)	16/23(65.6)	
Invasive mucinous predominant	46						
HER2 (1+)	7	0/7(0.0)	7/7(100)	0.239	0/7(0.0)	7/7(100)	0.205
HER2 (2+)	27	1/27(3.7)	26/27(96.3)		5/27(18.5)	22/27(81.5)	
HER2 (3+)	12	2/12(16.7)	10/12(83.3)		4/12(33.3)	8/12(67.7)	

Abbreviations: HER2, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization; amplification or mutation: +, Negative: −.

Table 3
Multivariate analyses of factors that might affect the presence of EGFR mutation.

Variable	Category	95% CI	*P* value
Gender	Female, vs. male	2.329 (0.741–15.434)	0.035
Age	<60 years/≥60 years	1.029 (0.454–6.326)	0.145
Menopause	Premenopausal/ postmenopausal	4.031 (0.613–10.129)	0.046
Stage	I + II/III + IV	0.373 (0.204–2.177)	0.340
Pleural invasion	Yes, vs. no	0.874 (0.018–0.894)	0.463
Lymph node metastasis	Yes, vs. no	0.725 (0.219–3.156)	0.733
Vascular invasion	Yes, vs. no	1.503 (0.228–3.427)	0.671
Histological subtype	Acinar predominant/ others	0.251 (0.062–0.749)	0.018
Expression of cytoplasmic Eβ	Negative, vs. positive	0.232 (0.169–0.943)	0.032
Expression of nuclear Eβ	Negative, vs. positive	0.742 (0.243–0.761)	0.548
Expression of nuclear Eα	Negative, vs. positive	2.547 (0.561–6.247)	0.446
Expression of PR	Negative, vs. positive	1.167 (0.221–6.173)	0.855
Expression of HER2	Negative, vs. positive	1.922 (1.520–3.271)	0.015

type accounting for 62.5% (5/8), followed by missense mutations with 25.0% (2/8). Furthermore, we detected one non-coding pathogenic somatic BRCA1 mutation (Table 4). Further analysis revealed that among patients harboring acinar histologic subtype (6/8, 75.0%) had the higher percentage of BRCA mutation than other subtypes (Table 4). Like high prevalence of germline BRCA mutations in triple-negative phenotype breast cancer, cytoplasmic ERβ, PR, and HER2 protein expression were also negative in lung adenocarcinoma, harboring germline or somatic mutations in the BRCA1/2 gene. However, patients with concurrent gBRCAm and mutations in classic lung cancer driver EGFR genes were also found in our cohort (Tables 1, 4).

4. Discussion

Most studies reported that the staining for ERα was localized to the cytoplasm, a few reported nuclear expression of the receptor in NSCLC tumors [3,26]. By contrast, some studies reported that ERs, especially ERβ probably increased proliferation, reduced apoptosis, and increased angiogenesis, ERα is either absent or is expressed at very low levels [5,27]. The rate of PR expression in lung tumors varies among reports [3-5]. We found that NSCLC tumors expressed both estrogen receptors (ERα and ERβ) and PR by immunohistochemical technique, the more significant expression of nucleus and extranuclear ERβ than ERα, PR. Considering the inconsistent results of previous studies, ER function may be different due to variations in location of ER expression (nucleus vs. cytoplasm), ER isoforms (ERα vs. ERβ), and epitopes that each anti-ER monoclonal antibody can recognize in lung cancers. These discrepancies may obscure the significance of hormone receptor expression status on patient clinicopathologic characteristics and prognosis. The distribution of steroid receptors ERβ in different subcellular compartments may well modulate signaling by genomic, nongenomic and/or ligand-independent pathways that contribute to lung adenocarcinoma progression [28,29]. We also showed that subcellular translocation of ERβ existed in tumorous and non-tumorous lung adenocarcinoma samples of the same case. Thus, treatments aimed to the activity of ERα, ERβ and/or PR may modulate tumor progression and thereby offer new approaches to treat lung adenocarcinoma. Further studies will also be required to understand the significance of extranuclear and nuclear

Table 4
Mutational profile of the lung adenocarcinoma patients with germline and somatic mutations in BRCA1/2.

Patient number	Gender	Age (years)	Histology Subtype	BRCA mutations					EGFR (mut)
				Gene	ACMG	Mutation type	Mutation	MF	
1	Female	56	Acinar	gBRCA1m	Class 5	Frameshift	exon24: c.5470_5477del8 p.(I1824Dfs*3)	49.57%	L858R
2	Female	72	Acinar	gBRCA1m	Class 4	Frameshift	exon14:c.4464delT p.(N1488Kfs*17)	66.74%	WT
3	Male	67	Acinar	gBRCA2m	Class 5	Frameshift	exon11:c.6373_6374insA p.(T2125Nfs*4)	55.69%	19Del
4	Male	69	Acinar	gBRCA2m	Class 5	Frameshift	exon11:c.5164_5165delAG p.(S1722Yfs*4)	47.32%	WT
5	Female	53	Papillary	gBRCA1m	Class 4	Missense	exon11: c.954_955insT p.(N319*)	54.38%	WT
6	Female	61	Solid	gBRCA1m	Class 5	Missense	exon11: c.1660G>T p.(E554*)	60.25%	WT
7	Female	71	Acinar	sBRCA1m	Class 5	Frameshift	exon20: c.5266_5267insC p.(Q1756Pfs*74)	3.06%	WT
8	Female	63	Acinar	sBRCA1m	Class 4	Non-coding mutation	intron16:c.4986+4A>G p.?	39.48%	WT

Abbreviations: gBRCAm, germline BRCA mutation; sBRCAm, somatic BRCA mutation; EGFR, epidermal growth factor receptor; MF, mutation frequency; WT, wild type.

expression patterns of steroid receptors, to pursue these alternatives for specific targets of sex steroid action in lung adenocarcinoma.

The assessment of HER2 gene amplification and protein expression has benefited for current breast cancer diagnosis and target therapy [24]. As expected, our result showed that 28.9% (185/640) HER2 expression in lung adenocarcinoma samples. Invasive mucinous adenocarcinoma and micropapillary predominant adenocarcinoma were frequently detected the presence of HER2 expression. However, amplification of HER2 was detected in only three cases (3/110, 2.7%) and 26 HER2 mutations in 110 cases were identified (23.6%). HER2 mutations are thought to be more clinically relevant than over-expression or gene amplification in NSCLC. HER2 mutations were found to be more prevalent in East Asian ethnicity, adenocarcinoma histology, females, and never-smokers [9]. Therefore, we conclude that systematic genotypic testing in this subgroup of lung adenocarcinoma patients should include detection of HER2 mutations. According to a previous analysis, HER2 mutations were present in a small proportion of NSCLC patients, namely 2% to 5% [8]. Inconsistent with our data, we report a mutation rate of 28.9% in Chinese never-smoking lung adenocarcinoma in the HER2 immunopositivity patients. The possible explanations of the high frequency of HER2 mutations in our study were selection of antibodies widely used for breast cancer research, association between predominant histological subtype and high percentage HER2 immunopositivity, differences in mutation detection methods (real-time PCR analysis vs. direct sequencing), and small number of patients, all of whom were Chinese.

A strong association has been reported between the expression of ERβ and EGFR mutations in adenocarcinoma of the lung [29,30]. Some studies also provide evidence of a functional interaction between the ER and EGFR pathways in lung cancer and support a rationale to use combined therapy. Thus, endocrine therapy could also be beneficial for EGFR-mut lung adenocarcinoma as a combination therapy with EGFR-TKI [31]. We have demonstrated that higher prevalence of cytoplasmic ERβ and HER2 expression were found in micropapillary predominant subtype and the invasive mucinous predominant subtype. The percentage of EGFR-mut group was significantly higher in acinar predominant invasive adenocarcinomas, compared with the other subtypes. The mutations in the EGFR gene were significantly associated with expression of cytoplasmic ERβ ($P = 0.032$) and HER2 ($P = 0.015$). Further study of interactions between ER, HER2 and EGFR signaling in NSCLC, as well as co-targeting the ER, HER2 and EGFR pathways in NSCLC, is also warranted. As in studies of nuclear receptor expression in breast cancer [23,24], a standardized and validated approach to the immunohistochemical detection and interpretation of ERα, ERβ, PR and possibly HER2 expression is needed for potential screening of human lung tumor specimens in the clinic. A major limitation of this study was the lack of survival data. Since the cases were collected from 2014 to 2019, survival data of these patients were far from maturity, and further research in this aspect is warranted.

The majority of gBRCAm in triple-negative breast cancer patients and are predominantly composed of BRCA1 mutations [32]. BRCA2 mutation predominance was observed in prostate cancer patients [33]. Previous study revealed that 1.03% of gBRCAm with a predominance of BRCA2 (49/64, 76.5%) in Chinese NSCLC [34]. In the present study, we analyzed the incidence of gBRCAm and sBRCAm in Chinese lung adenocarcinoma patients, according to the IASLC/ATS/ERS classification. Our study found 0.94% of gBRCAm with a predominance of BRCA1 (4/6, 66.7%) and 0.31% of sBRCAm with 2 all of BRCA1. Emerging inconsistent results with previous study attributed to selected patients with different histologic subtypes, smoking status and pathologic stage. Our study only focused on no smoking lung adenocarcinoma and included the early and advanced stages (stage I-IV). Interestingly, we revealed two patients with concurrent gBRCAm and somatic EGFR mutations, so it may have a tendency to respond well to EGFR-TKI and PARP inhibitors. Further studies are required to elucidate clinical significance of BRCA for guiding target therapy in lung adenocarcinoma. Meanwhile, the present study has certain limitation of lack of family history.

5. Conclusion

In summary, our data have showed that the micropapillary and invasive mucinous predominant subtype lung adenocarcinoma are more likely to have cytoplasmic ERβ and HER2 expression, compared with the other subtypes. Here, we also found that there was a close correlation between the expression of cytoplasmic ERβ, HER2 and the presence of EGFR mutations. Furthermore, we revealed somatic and germline BRCA mutations in patient lacking ERβ, PR, HER2 expression in lung adenocarcinoma. Understanding the role of ERβ, HER2, EGFR and BRCA pathways in lung adenocarcinoma is necessary to develop new preventative and treatment strategies, especially histological subtypes of lung adenocarcinoma in line with the IASLC/ATS/ERS classification.

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CRediT authorship contribution statement

Lin Zhong: Investigation, Writing-Original draft preparation, Validation. **Chunfang Zhang:** Investigation, Material preparation, data collection. **Wenting Jia:** Formal analysis, data curation, Software. **Pengxin Zhang:** Conceptualization, Methodology, Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare no competing financial interests.

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