NTRK, RET, ROS1, and ALK Gene Fusion in HER2 Immunohistochemistry 2+ Breast Carcinoma

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Abstract

Objective

HER2 immunohistochemistry (IHC) 2+ breast cancer patients need to determine the final HER2 status by fluorescence *in situ* hybridization (FISH) for selection of suitable treatment options. Although the HER2-positive cases can benefit from the anti-HER2 targeted therapy, it only made a small proportion of this group, so finding more targeted therapy methods is necessary. *NTRK*, *RET*, *ROS1* and *RET* gene fusions have been fully investigated in non-small cell lung carcinoma and are subject to targeted therapy in clinical practice and trials. However, there are only few reports investigating these four fusion genes in breast cancer. Our study is designed to evaluate the four fusion genes in HER2 IHC 2+ breast cancer patients to find an alternative treatment option.

Methods

One hundred and seventy-seven tissue samples were included. IHC was employed to assess ALK and NTRK protein levels. FISH probes specific for *HER2*, *ALK*, *NTRK1*, *NTRK2*, *NTRK3*, *ROS1* and *RET* were used.

Results

The HER2-positivity rate of all HER2 IHC 2+ cases were 5.7%. The total fusion rate of the four oncogenes was 3.95% in HER2 IHC 2+ breast cancer patients. The fusion-positive patients were prone to be ER/PR/HER2 IHC triple negative (P=0.01) and were associated with poorly differentiated tumor (P=0.005). The *NTRK*, *RET*, *ROS1*, and *ALK* fusion rate was 0.56%, 1.13%, 1.13%, 1.13%, respectively.

Conclusions

NRTK, *RET*, *ROS1*, and *ALK* fusion rearrangements were detected in triple-negative breast carcinoma patients which can provide patients with alternate treatment opportunities in clinical practice.

Highlights

- HER2 IHC 2+ breast cancer patients need to determine the final HER2 status by FISH.
- Finding more targeted therapy methods for the breast cancer patients is necessary.
- *NRTK*, *RET*, *ROS1*, and *ALK* gene fusion rate was 3.95% in our cohort.
- The fusion-positive patients were prone to be ER/PR/HER2 IHC triple negative.

Introduction

Breast cancer is classified into molecular subtypes according to the expression of markers such as estrogen receptor-alpha (ER-α), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Each subtype is characterized by distinct clinical features, including response to treatment, pattern of recurrence, and survival. The determination of HER2 expression or amplification is pivotal during clinical practice, as it is a predictive marker for potential responsiveness to targeted therapies in breast cancer patients. The expression of HER2 is routinely determined by immunohistochemistry (IHC) and IHC results are divided into three categories based on scores: HER2-negative (0, 1+), HER2-equivocal (2+), and HER2-positive (3+). According to the recommendation of the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP), samples of HER2 IHC 2+ form a special group that need further FISH analysis for the final determination of HER2 status [1]. The final hormone and HER2 status is pivotal for the selection of suitable treatment methods in the HER2 IHC 2+ patients. For example the hormone positive and HER2 negative patients in HER2 IHC 2+ group can receive the endocrine therapy, the HER2 positive patients in this group can accept the anti-HER2 targeted therapy. While the only approved treatment method for the hormone negative and HER2 negative patients (triple negative) patients is cytotoxic chemotherapy. Approximately 17% of HER2 IHC 2+ patients are classified as HER2-positive cases after the confirmatory fluorescence *in situ* hybridization (FISH) test and this subgroup of patients can benefit from targeted therapy[2]. The other patients are devoid of targeted therapy methods in the HER-2 IHC 2+ patients. Additionally, advanced or metastatic hormone receptor-negative and HER2-negative breast cancer patients have no effective choice of treatment, development of novel drug targets offers alternative treatment opportunities.

RTK (receptor-tyrosine kinase) gene rearrangement can lead to ligand-independent activation of the oncogenic signaling pathway and increase proliferation and survival of tumor cells. RTK rearrangement is a rare event in solid tumors. Approximately 250,000 new cases of invasive breast cancer are diagnosed in the U.S. annually, there are approximately 5000 new cases of RTK fusion-positive patients each year that may benefit from the corresponding targeted therapy [3]. As these biomarkers provide alternative treatment options for advanced or refractory breast cancer patients, it is worth the effort to investigate RTK fusion genes in breast carcinoma.

RTKs such as neurotrophic tropomyosin-related kinases (NTRKs) play an important role in neuronal development and exhibit limited expression in the nervous system after embryogenesis. *NTRK* codes for a family of genes: *NTRK1* (also known as *TRKA*), *NTRK2* (also known as *TRKB*), and *NTRK3* (also known as *TRKC*). *NTRK* is a widespread cancer biomarker targeted for therapeutics, and oncogenic fusions involving *NTRK1*, *NTRK2* and *NTRK3* have been reported in a variety of malignancies and represent key mechanisms of oncogenic TRK activation [4]. Multiple fusion partners of *NTRK* genes have been described. The best known form is *ETV6-NTRK3*, which is a typical gene alteration in secretory carcinomas of the breast, salivary glands, and congenital fibrosarcoma [5-7]. However, the reported NTRK fusion rates in the non-secretory type breast carcinoma are variable and not fully investigated. *ROS1*, a proto-oncogene expressed in various tumor cell lines, is a regulator of cellular signal transduction pathway that mediates cell proliferation and migration and cell-to-cell communication. *ALK* gene fusion was first reported in anaplastic large cell lymphoma in 1994 [8, 9]. Thereafter, this fusion oncogene was found to be associated with the

development of diverse tumor types of different lineages, including lung cancer, inflammatory myofibroblastic tumors, Spitz tumors, renal carcinoma, thyroid cancer, digestive tract cancer, breast cancer, leukemia, and ovarian cancer [10].

The new U. S. Food and Drug Administration (FDA)-approved NTRK-targeted drug elecintinib is also effective in *ALK* and *ROS1* fusion-positive cancer patients. RET (Rearranged during transfection) is proto-oncogene which can be found in 6.8% of papillary carcinoma and 1-2% of NSCLC patients [11, 12]. Additionally, cancers harboring RET fusion are collectively termed as 'REToma', similar to the termination of 'ALKoma' for tumors with ALK alterations [13, 14]. Thus, considering the opinion that cancer may be treated with TKIs (tyrosine kinase inhibitors) irrespective of the tissue of origin, it is worthy to explore the specific fusion rates of different cancer types before applying targeted therapy. In our study, we focused on evaluating the rearrangement of four different RTKs, *NTRK*, *RET*, *ROS1* and *ALK*, in patients with HER2 IHC 2+ breast cancer.

Materials And Methods

Patient information

One hundred seventy-seven patients were enrolled in our study, who underwent surgery between October 2016 and January 2018 at Peking Union Medical College Hospital (Beijing, China). All the cases were diagnosed according to the morphology of the hematoxylin and eosin (HE) staining of the tumor sample and immunohistochemistry results. The secretory type of breast carcinoma was excluded from our cohort. Two experienced pathologists reviewed the HE slides to confirm the final diagnosis.

The clinical and pathological information of the subjects, including age, sex, tumor sizes, ER-α, PR, HER2 and Ki-67 index, were collected from digital clinical archives and pathological reports.

This study was approved by the institutional review board of Peking Union Medical College Hospital.

Tissue microarray construction

The selective areas of representative morphology of the HE slides were labeled. The corresponding formalin-fixed paraffin-embedded (FFPE) primary tumor samples were obtained from the Department of Pathology. The tissue microarray construction machine (Quick-Ray UT-06, UNITMA) was used and two core-tissue biopsies of 2.0mm diameter were collected for each sample. Each block contained 6 cases of HER2-negative and 2 cases of HER2-positive cases, serving as the negative and positive control, respectively. This tissue microarray block was evaluated by FISH analysis.

Immunohistochemistry

NTRK immunohistochemical staining employed the antibody clone EPR17341 (Roche, Tusan, US) to assess NTRK1, NTRK2, and NTRK3 protein expression in the FFPE samples. Positive result was defined as staining above background in at least 1% of tumor cells in any pattern, including membranous, cytoplasmic, perinuclear, or nuclear. ALK D5F3 (Roche, Cambridge, MA) antibody was used to examine ALK protein expression. Only strong, brown cytoplasmic staining in the tumor cells was deemed as positive. The other antibodies used in the study include ER (clone SP1, Ventana Medical System, Inc., Tucson, AZ, USA), PR (clone IE1, Ventana Medical System, Inc., Tucson, AZ, USA), HER-2 ((clone 4B5, Ventana Medical System, Inc., Tucson, AZ, USA), and Ki-67(cloneUMAB107, OriGene Technologies Inc., USA). ER and PR was deemed as positive if there are brown nuclear staining in above 1% of the tumor cells. Ki-67 was also considered as positive when the tumor nuclear stains. Her-2 2+ is defined as incomplete circumferential membrane staining and /or weak/ moderate staining within >10% of tumor cells, or complete and intense circumferential membrane staining within ≤10% of the cells according to the suggestion of 2013 ASCO/CAP guidelines for qualification of HER-2 status in invasive breast carcinoma.

FISH

FISH was performed on 4-um slides using the Thermo-Brite Elite automated FISH slide prep system (Leica, Richmond, CA, USA) with the FISH break apart probes used in our study include Vysis ALK Break Apart FISH Probe (Abbott Molecular, Des Plaines, IL, USA), NTRK1, NTRK2, NTRK3 Break Apart FISH Probe (ZytoVision GmbH, Bremerhaven, Germany), 6q22 ROS1 Break Apart FISH Probe (Abbott Molecular, Des Plaines, IL, USA), and SPEC RET Dual Color Break Apart Probe (ZytoVision GmbH, Bremerhaven, Germany). One hundred tumor nuclei per case were calculated and the case was considered positive (rearranged) if 15% or greater tumor cell nuclei were rearranged. Different evaluation criteria were set for different probes. For *ALK*, cell was considered positive if there was a split of two or more signal widths apart between the orange and green signals or there was a single orange signal without a corresponding green signal in combination together with a fused and/or split signal. For *ROS1*, cell was considered positive if there was a split of two or more signal widths apart between the orange and green signals or there was a single green signal without a corresponding orange signal in combination with a fused and/or split signal. For *RET*, cell was considered positive if there was a split of one or more signal widths apart between the orange and green signals or there was a single green signal without a corresponding orange signal in combination with a fused and/or split signal. For *NTRK1/2/3*, cell was considered positive if there was a split of one or more signal widths apart between the orange and green signals.

HER-2 FISH test was performed with a PathVysion HER2 DNA probe kit (Abbott Molecular, Des Plaines, IL, USA) according to the standard protocol. FISH results were evaluated according to the ASCO/CAP HER2 testing guidelines: it was considered positive when the ratio of HER2/CEP17 ≥2.0 or the average HER2 signal/tumor cell ≥6.0 with a ratio of HER2/CEP17 <2.0; HER2 negative was determined when the ratio of HER2/CEP17<2.0.

Results

A total of 177 invasive breast carcinomas of HER2 IHC 2+ status was included in our study. The age of the patients ranged from 26 to 88 years (median 54 years). The histological subtypes included 171 (97.2%) cases of invasive carcinomas of no special types and 5 (2.8%) cases of special type breast carcinoma, which included 3 cases of micropapillary carcinomas, 1 case of papillary carcinoma, and 1 case of mucinous carcinoma. According to the morphology and IHC results, none of them were secretory type breast carcinoma. The median tumor size was 2.0 cm (ranging from 0.5 cm to 9 cm).

All the above patients were HER2 2+ as determined by IHC. After further evaluation by FISH, 10 (5.7%) of the 177 patients were diagnosed as HER2-positive, and 167 (94.3%) of them were HER2-negative. The median Ki-67 index was 25% (ranging from 2% to 90%).

The total fusion rate of *NTRK*, *ROS1*, *RET*, and *ALK* genes was 3.95%. NTRK IHC result was positive in 5 cases, and 1 of them was further confirmed to be NTRK1-rearranged, using FISH analysis. The rest of the cases were devoid of break apart signal in the FISH analysis. The final *NTRK* fusion rate was 0.56%. Two of the three *ALK* fusion-positive samples detected by IHC were confirmed to be positive by FISH test. The final *ALK* fusion rate was 1.13%. There were two (1.13%) *RET* fusion-positive and two (1.13%) ROS1 fusion-positive patients in our cohort (Figure 1). Detailed information regarding the fusion-positive patients is summarized in Table 1.

The fusion rearrangement was associated with ER negative (p=0.011), PR negative (p=0.002), and triple negative (p=0.01) status. The fusion-positive tumors were poorly differentiated compared with the fusion-negative group (p=0.005). There was no significant difference between groups with respect to patients' age, tumor size, tumor type, HER-2 status, or Ki-67 index (Table 2).

Discussion

NRTK, *RET*, *ROS1*, and *RET* fusion rearrangements are the most common genomic aberrations and have been fully investigated in non-small cell lung carcinoma (NSCLC). RTK-targeted therapy is available in clinical practice or trials. However, information regarding RTK fusion in breast carcinoma is limited. In our study, we found a fusion-positive rearrangement rate of 3.95% for the above genes in HER2 2+ breast cancer patients, and these patients were associated with ER/PR/HER2-negative status and poorly differentiated tumor type compared with the fusion-negative group. To our knowledge, this is the first report showing the presence of RTK-fusion oncogenic alterations in breast cancer. The detection of the four RTK fusion genes in the HER2 2+ patients can provide this special group of patients an alternative treatment option, especially for the triple negative breast carcinoma patients who lack an effective and less toxic treatment options in this group.

NTRK is proved to be a pan cancer marker and *NTRK*-targeted therapy has been efficient regardless of tumor types or fusion partners. Moreover, the *NTRK* inhibitor entrectinib is also effective in *ROS1* and *ALK* fusion-positive tumors. The *RET, ROS1* and *ALK* fusion-positive NSCLC patients have similar clinical characteristics and were likely to be young and female and have non-smoker adenocarcinoma without *EGFR, BRAF*, and *KRAS* mutations. With the popularity of the terms '*ALK*oma' and 'REToma' and effective treatment outcomes in fusion-positive tumors other than NSCLC, it is important to note that apart from fusion rates of these genes, clinical features of fusion-positive patients should be taken into consideration before testing. As there are no effective treatment methods for triple-negative breast cancer patients currently, the finding that fusion-positive breast cancer patients are prone to be triple-negative is valuable to clinical practitioners. Furthermore, the gene fusion in the breast carcinoma is a rare event, enrichment of the targeted population is pivotal, and testing the fusion genes in the triple negative patients could be a practical choice. Validation in a larger group of breast carcinoma patients, especially in the triple-negative category, will be needed in the future.

Larotrectinib and entrectinib are FDA-approved drugs that can be used in the treatment of solid tumors harboring *NTRK* gene fusion. It is pivotal to identify the fusion status of individuals to apply the correct targeted therapy. In our study, a positive *NTRK*-fusion rate of 0.56% was found in the HER2 2+ patients. The reported *NTRK*-fusion rates in literature range from 0% to 0.34% in the non-secretory type of breast carcinoma [15, 16]. Despite the differences in the selected HER2 2+ patient population, in terms of ethnicity, the result of the present study is similar to the previous ones. Further studies on examining the rate of NTRK fusion in larger number of breast cancer patients of Asian origin are needed before a decision of rate of NTRK-fusion is made.

RET gene, a driver oncogene, is a therapeutic target and can be found in 6.8% of papillary carcinoma and 1-2% of NSCLC patients [11, 12]. There are a few studies reporting an incidence of 0.17% of *RET* gene fusion in breast carcinoma. The main reason for the higher RET fusion rate in the present study (1.13%) may be due to differences in the enrolled patients. HER2-positive patients were the focus of the present study while in previous study, patients with available ER information were included and the RET fusion positive patients were prone to have a negative ER status. Furthermore, in the present study, two patients were ER positive and ER negative, respectively. The chromosomal rearrangement of *RET* leads to activation and formation of *RET* fusion protein which is capable of auto dimerization. Other *RET* gene alterations include mutation and amplification. The *RET* mutations in breast cancer patients are reported to be prone in young female patients and confer resistance to hormone therapy [17]. The *RET* fusion-positive patients in our study are all young patients of age 48 all young patients of age 48 and 26 at diagnosis, similar to the clinical characteristics of RET mutation patients. There was a preliminary report which showed the use of a combination of aromatase inhibitor and *RET* inhibitor resulted in better efficacy as opposed to aromatase inhibitor alone in breast cancer cell lines [18]. The *RET* inhibitor also showed promising results in refractory ER+/HER2+ breast cancer patients [19]. Although the fusion rate of *RET* gene in breast cancer is much lower than that of papillary thyroid carcinoma (PTC), it is comparable with that of NSCLC. Therefore, it is important to identify *RET* fusion-positive patients and provide alternative treatment options.

The average frequency of *ALK* fusion is approximately 5% in NSCLC and several FDA approved targeted therapy drugs have been used in NSCLC as a first line treatment option [20].According to a report on gene rearrangements in solid tumors, *EML4-ALK* fusion was found in 5 of 209 breast carcinomas (2.4%) [21]. The fusion rate of *ALK* in our study is 1.13%, wherein the two individuals are triple negative breast cancer patients. The ALK kinase domain shares a similar sequence identity with that of the proto-oncogene *ROS1* (50% similarity). The ALK inhibitor, crizotinib, is also effective in *ROS1* fusion-positive NSCLC patients. Inhibition of ROS1 and its phosphorylation level lowers the *in vitro* alcohol-induced breast cancer cell proliferation and growth [22]. Another study showed that ROS1 inhibitors produce synthetic lethality in E-cadherin-deficient cell line and anti-tumor effects in *in vivo* models of breast cancer [23]. *ROS1* gene

rearrangements rate is approximately 1–2% in NSCLC patients [24, 25], and presence of ROS1 fusion-positive patients in cholangiocarcinoma, glioblastoma, ovarian, gastric, and colorectal cancers is also reported [26]. Similarly, in the present study, two *ROS1* fusion-positive patients (1.13%) were identified. The two tumors were poorly differentiated with invasion ductal carcinoma, not otherwise specified. One patient was ER+/HER2+ while the other was ER+/HER2-.

Our cohort included cases with HER2 expression with an IHC score of 2+, thereby narrowing down the study to specific subtypes of breast cancer. Thus, this study may not reflect the true incidence of RTK fusion in the general population. Owing to the retrospective nature of this study, we analyzed the fusion rate of RTK genes independent of drug efficacy with regard to gene rearrangement characteristics. Nevertheless, this study shows that RTK fusion-positive status can provide breast cancer patients with alternate treatment opportunities in clinical practice.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) Version 20.0 Software (SPSS Inc., Chicago, IL) was used to analyze the data. Chi-square or Fisher's exact tests were employed to analyze the categorical variables or continuous variables as appropriate. All p values are reported as two-sided with the p values < 0.05 being considered statistically significant.

Abbreviations

IHC: immunohistochemistry; FISH: fluorescence *in situ* hybridization; ER-α: estrogen receptor-alpha; PR: progesterone receptor; HER2: epidermal growth factor receptor 2; ASCO: American Society of Clinical Oncology; CAP: College of American Pathologists; n NTRKs: eurotrophic tropomyosin-related kinases; FDA: Food and Drug Administration; RET: Rearranged during transfection; FFPE: formalin-fixed paraffin-embedded; NSCLC: non-small cell lung carcinoma;

Declarations

Authors' contributions

SW and XS performed experiments and data analysis and wrote paper; YJ and KL reviewed all the cases together; YL and XZ did the FISH and data analysis; ZL conceptualized the study design and paper writing. All authors read and approved the final manuscript.

Data availability statement

All datasets analyzed for this study are included in the article

Compliance with ethical standards Conflict of interest The authors declare that they have no competing interests.

Ethics statement All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The retrospective study was approved by the institutional review board of Peking Union Medical College Hospital with a waiver for the need to obtain informed consent.

Informed consent This article does not contain any studies with human participants or animals. The entire study protocol was approved by the Medical Ethical Committee of the Peking Union Medical College Hospital.

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Tables

Table 1 Detailed information of the fusion-positive patients.

Fusion type	Age	tumor size	Differentiation	ER	PR	HER2	Ttripple negative	Ki-67 index (%)
NTRK1	45	1.3	poor	0	0	0	1	70
ROS1	77	1.8	poor	1	0	1	0	40
	45	1.5	poor	1	0	0	0	30
RET	48	1.8	poor	0	0	0	1	60
	26	2	poor	1	1	0	0	20
ALK	63	1.2	poor	0	0	0	1	60
	51	1.9	poor	0	0	0	1	25

Table 2 Comparation between the fusion-positive and negative patients.

		Total fusion		P	NTRK		RET		ROS1	
		Positive	Negative		Positive	Negative	Positive	Negative	Positive	Negative
Age (years old)		50.71±15.95	54.62±12.62	0.427	45	54.52±12.75	37.00±15.56	54.67±12.61	61.00±22.63	54.39±12.67
Tumor size (cm)		1.71±0.49	2.23±1.33	0.309	1.3	2.22±1.31	2.00±0.00	2.21±1.32	2.00±0.00	2.21±1.32
Tumor type	NOS	7	164	1	1	170	2	169	2	169
	Special type	0	5		0	5	0	5	0	5
Tumor differentiation	Well	0	22	0.005	0	22	0	22	0	22
	Moderate	0	78		0	78	0	78	0	78
	Poor	7	63		1	69	2	68	2	68
ER	Positive	3	147	0.011	0	150	1	149	2	148
	Negative	4	23		1	26	1	26	0	27
PR	Positive	1	128	0.002	0	129	1	128	0	129
	Negative	6	42		1	47	1	47	2	46
HER-2	Positive	1	9	0.339	0	10	0	10	1	9
	Negative	6	161		1	166	2	165	1	166
Ttriple negative	Y	4	22	0.01	1	25	1	25	0	26
	N	3	148		0	151	1	150	2	149
Ki-67 index		0.44±0.20	0.30±0.23	0.118	0.7	0.30±0.23	0.40±0.28	0.30±0.23	0.35±0.71	0.30±0.23

Figures

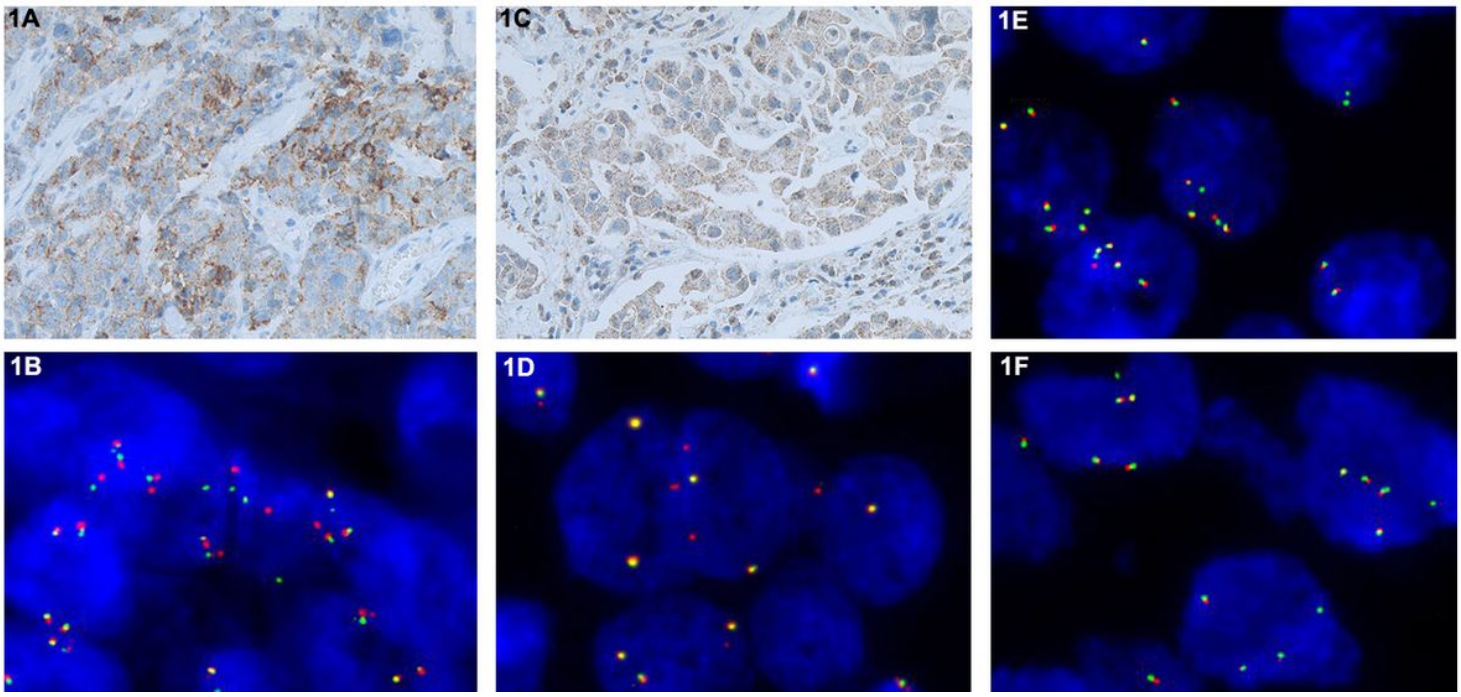

Figure 1

A, the positive immunohistochemical stain of NTRK (X200). B, the positive FISH result of NTRK (X400), there were split orange and green signals in the tumor cells. C, the positive immunohistochemical stain of ALK (X200). D, the positive FISH result of ALK (X400), there were split orange and green signals in the tumor cells. E, the positive FISH result of RET (X400). F, the positive FISH result of ROS1 (X400).