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Efficacy of neratinib in the treatment of HER2/neu-amplified epithelial ovarian carcinoma in vitro and in vivo

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Abstract

Epithelial ovarian carcinoma is the most lethal of gynecologic malignancies. There is a need to optimize the currently available treatment strategies and to urgently develop novel therapeutic agents against chemotherapy-resistant disease. The objective of our study was to evaluate neratinib's preclinical efficacy in treating HER2-amplified ovarian cancer. Neratinib's efficacy in treating HER2-amplified ovarian cancer was studied in vitro utilizing six primary tumor cell lines with differential HER2/neu expression. Flow cytometry was utilized to assess IC₅₀, cell signaling changes, and cell cycle distribution. Neratinib's in vivo efficacy was evaluated in HER2-amplified epithelial ovarian carcinoma xenografts. Three of six (50%) ovarian cancer cell lines were HER2/neu-amplified. Neratinib showed significantly higher efficacy in treating HER2/neu-amplified cell lines when compared to the non-HER2/neu-amplified tumor cell lines (mean $\pm$ SEM IC₅₀: 0.010 μ M $\pm$ 0.0003 vs. 0.076 μ M $\pm$ 0.005 $p < 0.0001$). Neratinib treatment significantly decreased the phosphorylation of the transcription factor S6, leading to arrest of the cell cycle in G0/G1 phase. Neratinib prolonged survival in mice harboring HER2-amplified epithelial ovarian carcinoma xenografts ($p = 0.003$). Neratinib inhibits proliferation, signaling, cell cycle progression and tumor growth of HER2-amplified epithelial ovarian carcinoma in vitro. Neratinib inhibits xenograft growth and improves overall survival in HER2/neu-amplified ovarian cancer in vivo. Clinical trials are warranted.

Keywords

Neratinib; Epithelial ovarian carcinoma; SCID mice; HER2/neu

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of mice were followed. Ethical approval for involving mice in our study was obtained from Yale Institutional Animal Care and Use Committee (IACUC) which granted the ethical approval after review. The policies set forth by the IACUC at Yale University were followed while housing and treating the mice.

Human and animal rights This article does not contain any studies with human participants performed by any of the authors.

Introduction

Epithelial ovarian, peritoneal, or fallopian carcinoma (EOPFC) is the most lethal of gynecologic malignancies. According to the National Cancer Institute [Surveillance, Epidemiology, and End Results (SEER) Data] 22,280 EOPFC will be diagnosed and 14,240 will die from the disease in 2016 [1]. EOPFC remains a serious challenge, with the highest case–fatality ratio of all gynecologic cancers. The core elements of primary therapy for EOPFC, including cytoreductive surgery and platinum-based chemotherapy, remain largely unchanged over the last 25 years. Despite surgical and medical treatment advances, metastatic/recurrent ovarian carcinoma is still an incurable disease [2, 3]. For this reason, it is of outmost importance to explore potential molecular targets that may improve survival of ovarian cancer patients. Accordingly, potential therapeutic targets have been identified and are being tested in various clinical trials [2]. Among such targeted therapies, tyrosine kinase inhibitors have received special attention.

Recent focus of research has been to understand the genetic landscape of different tumors including EOPFC through next generation sequencing. Along these lines, defining the genetic landscape for a number of EOPFC may allow providers to directly target altered pathways [4, 5]. In this regard, a number of human tumors have been identified to rely on HER2/neu mutations and/or amplifications [6, 7], which then led to the development of tyrosine kinase inhibitors. Observed rates of HER2 overexpression/amplification have been reported to range from 8 to 66% in ovarian carcinoma [8]. Overexpression of HER2/neu is linked to a more aggressive tumor phenotype which might result in worse prognosis in a number of cancers including EOPFC [9–12]. This has been explained by the increase in the number of surface HER2/neu receptors of tumor cells with HER2/neu-amplification which leads to proliferation of the tumor by increasing homo- and hetero-dimerization [12, 13]. Neratinib (HKI 272) is an irreversible inhibitor of ErbB1 and HER2/neu and was developed to inhibit the signaling pathways that are induced by receptor dimerization.

The objective of current study was to evaluate the effects of neratinib (HKI-272) on cell viability, cell cycle progression, and downstream signaling pathways in HER2/ neu-amplified EOPFC both in vitro and in vivo. Neratinib might be a novel treatment option for patients harboring chemotherapy-resistant EOPFC.

Materials and methods

Establishment of cell lines and HER2 expression analysis

Prior to surgical staging, patients were consented for tumor banking. This was carried out through a Yale University institutional review board approved HIC, which was designed in accordance with the Helsinki Declaration. At the time of frozen section, small portions of viable tumor were collected for processing and establishment of primary cell lines. Tumor samples were processed and de-identified as previously described [14, 15]. Briefly, tumors were processed using a scalpel for mechanical disruption in an enzymatic solution of 0.14% collagenase type 1 (Sigma) and 0.01% DNase (Sigma, 2000 KU/mg) in RPMI 1640. Dissociated tumor pieces were allowed to incubate in the same solution while stirring for 1 h at room temperature. The samples were then washed with RPMI and plated in Petri dishes in

Media (RPMI containing 10% FBS, 1% penicillin with streptomycin and 1% fungizone). They were kept in an incubator at 37 °C with 5% CO₂. Cell culture was continually checked and monitored for growth.

Neratinib's efficacy was tested using tumor cell cultures established as cell lines using previously published protocols [15]. FISH and IHC testing were utilized to determine HER2/neu expression of each primary tumor [13]. Briefly, cell lines that showed weak staining in <10% of cells were considered 0, those with staining in 10–30% of cells were classified as 2+, while cell lines with uniform staining in >30% of cells were classified as 3+ [15]. HER2/neu expression was determined by FISH assays in primary cell lines [16].

Drug

10 mM stock solution of neratinib (HKI-272) was created by dissolving HKI-272 in DMSO. Solutions of different concentrations were then created by diluting the stock solution: 0.2, 0.02, 0.01, 0.002 and 0.0004 µM. Sterile water with 0.5% methylcellulose (Sigma Life Sciences, St. Louis, MO) and 0.4% polysorbate 80 were used to dissolve neratinib for in vivo experiments [15].

Determination of IC₅₀

Briefly, six well plates were used to plate 30,000 cells per ml and cells were treated with the above-mentioned concentrations of neratinib (i.e., 0.2, 0.02, 0.01, 0.002 and 0.0004 µM) after 24-h incubation. The six well plates were allowed to incubate for 72 h. Once the contents of each six well were harvested, propidium iodide (Sigma Life Sciences, St. Louis, MO) was utilized to stain the cells and flow cytometry was the technique employed to count the cells. The number of viable cells in each well was normalized to the number of viable cells in the control well. The log base 10 of drug concentration in each well was compared to the percentage of viable cells using a non-parametric three parameter regression in order to calculate the IC₅₀ of each cell line. Prism 5 software (GraphPad Prism Software Inc., San Diego, CA) was used to calculate IC₅₀ data, which are presented as mean ± standard error of the mean. Each experiment was repeated three times.

Analysis of cell cycle

Cells were plated in a six well plate at a concentration of 30,000 cells/ml and were incubated overnight followed by treatment with scalar neratinib concentrations, as previously detailed. After 48 h of incubation, the cells were harvested and fixed in 70% ethanol for 30 min on ice. Once the cells were washed with PBS, they were treated with 100 µl of ribonuclease (conc 100 µg/ml) in PBS for 5 min at room temperature. Propidium iodide (conc 50 µg/ml) was added to each sample so that a final volume of 500 µl was reached. Flow cytometry was utilized to read cell cycle, while FlowJo was used for the final analysis (FlowJo, Ashland, OR). The data from the cell cycle analysis experiments are expressed as mean ± standard deviation (mean ± SD) [15].

Effects on S6 phosphorylation

A HER2-amplified cell line was selected in order to study neratinib's effects on S6 phosphorylation. Thirty thousand cells were plated per 1 ml in six well plates. In order to

evaluate maximal effects of neratinib on S6 phosphorylation, dose–response experiments using 0.02 (IC₅₀), 0.065 (half the physiologic dose in human), and 0.133 (physiologic dose in human) μ M of neratinib were carried out at 24 h [15]. The mean fluorescent intensity (MFI) was then compared between treated and untreated control cells which were compared in terms of their mean fluorescent intensity (MFI), and the data from phosphorylation experiments are demonstrated as mean $\pm$ standard deviation [15].

Efficacy in vivo

Each mouse was injected intraperitoneally with seven million cells of OSPC ARK-1 suspended in a 50%/50% mixture of 150 μ l of matrigel (BD Biosciences) and PBS. Fourteen-day period was allowed for tumor establishment and oral gavage with the treatment drug was administered subsequently, as previously described [17]. The mice were given neratinib 40 mg/kg/day for 5 days a week for eight consecutive weeks [18], and mouse weights were watched closely by recording the same at least twice per week. No signs of general toxicity were seen and mice were sacrificed when they were found in a moribund condition or if mice appeared in poor health. Ethical approval for involving mice in our study was obtained from Yale Institutional Animal Care and Use Committee (IACUC) which granted the ethical approval after review. The policies set forth by the IACUC at Yale University were followed while housing and treating the mice.

Statistics

Neratinib's efficacy was compared between EOPFC cell lines with and without HER2-amplification. One-way analysis of variance and two-tailed Student's *t* test were utilized to compare the IC₅₀ values of the eight cell lines and grouped mean IC₅₀ values, respectively. Two-tailed Student's *t* test was employed to compare cell cycle data and mean fluorescence intensities of phosphorylated S6 between cell lines with and without HER2-amplification. Overall survival of HER2-amplified xenografts was analyzed with a Kaplan–Meier curve and log rank test. Prism 6 software (GraphPad Prism Software Inc., San Diego, CA, USA) was utilized for all statistical analysis, considering a *p* value of <0.05 statistically significant.

Results

Evaluation of HER2/neu expression and neratinib IC₅₀ in primary ovarian cancer cell lines

Characteristics of the cell lines and of the patients are presented in Table 1. The effects of neratinib was evaluated using three cell lines with HER2/neu-amplification and three non-amplified cell lines with similar growth rates. Compared with the non-amplified cell lines, those with HER2/neu-amplification were significantly more susceptible to neratinib growth inhibition, *p* < 0.0001 (Fig. 1a). Similarly, the mean IC₅₀ for HER2-amplified cell line group was significantly lower than the IC₅₀ for non-amplified group, mean $\pm$ SEM IC₅₀: 0.010 μ M $\pm$ 0.0003 versus 0.076 μ M $\pm$ 0.005 (*p* < 0.0001), respectively (Fig. 1b). In other words, there was decreased in vitro cell proliferation when HER2/neu driver pathway was inhibited.

Cell cycle analysis

In order to further substantiate and support our above-mentioned results, we analyzed downstream signaling and cell cycle. Cells were plated and incubated with scalar amount of

neratinib for 24 h. As representatively shown in Fig. 2, neratinib caused arrest in the G0/G1 phase of the cell cycle at both 0.065 μM ($p = 0.02$) and 0.133 μM ($p = 0.01$), likely leading to apoptosis of tumor cells (Fig. 2).

Analysis of downstream signaling

The data from the above-mentioned IC50 and cell cycle analysis experiments clearly suggest that neratinib causes cell cycle arrest and decreases HER2-amplified tumor survival with very low concentrations of the drug. We then analyzed the downstream effects of neratinib on the transcription factor S6, in order to evaluate the mechanism of action (MOA) of neratinib and to determine whether the MOA is via HER2/neu pathway inhibition. As representatively shown in Fig. 3, we found neratinib to cause a significant reduction in the phosphorylation of S6 at all dose tested in dose–response experiments at 24 h (i.e., 0.02 μM , 0.065 μM , and 0.133 μM , Fig. 3).

Neratinib treatment of OSPC ARK-1 xenografts in mice

Xenografts were established over a 14-day period as previously described [17]. The mice were then divided into two groups, namely neratinib and vehicle. The mice in the vehicle group (i.e., control) received 100 μl water containing 0.5% methylcellulose and 0.4% polysorbate 80 for 5 days per week by oral gavage. The treatment group mice received neratinib 40 mg/kg/day dissolved in vehicle by oral gavage for 5 days per week [18]. Mouse weights were recorded twice weekly over a 60-day period. Mice gained weight at a similar rate compared to untreated mice and tolerated the treatment well (data not shown). Treatment with neratinib significantly inhibited growth of the tumor and improved overall survival in xenografts with HER2-amplification, $p = 0.0003$ (Fig. 4).

Discussion

Given the dismal prognosis of advanced ovarian cancer patients, the importance of tyrosine kinases in initiating/ progressing carcinogenesis and the gains of targeting them in multiple human cancers [19], we evaluated the activity of neratinib (HKI-272), an irreversible pan c-erb inhibitor, against multiple primary ovarian cancer cell lines with differential expression of HER2. We demonstrate for the first time that HER2/neu-amplified EOPFC cell lines treated with neratinib in vitro show a significant decrease in the transcription factor S6 phosphorylation. Furthermore, we observed a significant arrest of the cell cycle in the G0/G1 phase which was brought about by changes in cell signaling and inhibition of the tumor's driver pathway HER2/ neu. Similarly, the inhibition of the driver pathway allows for improved survival in vivo in xenografts harboring aggressive ovarian cancer.

HER2-neu encodes a transmembrane protein tyrosine kinase receptor and is involved in the occurrence and development of many types of cancers, including ovarian cancer [20]. Dysregulated HER2-neu signaling in EOPFC results from either gene amplification or overexpression, which leads to faster cell proliferation, DNA damage, and increased colony formation [21]. HER2-amplification/ overexpression is associated with poor prognosis in several cancer types [22, 23] and its prognostic value in ovarian cancer has been reported by many investigators [24, 25]. HER2 positivity in EOPFC varies in the literature from 8 to

66% [8]. The link between HER2 receptor overexpression and patient outcomes [26], complemented with the potent oncogenic role of HER receptors in preclinical models, established the bases for the development of agents targeting TKIs and HER receptors for the treatment of cancer patients with high HER receptor functionality [27].

Pivotal trials reported in 2005 showed that patients with HER2-positive early-stage breast cancer had improved survival when trastuzumab was added to standard chemotherapy [28–31]. However, one third of patients still develop recurrent disease despite the treatment with trastuzumab [28, 30, 32]. In an effort to overcome the trastuzumab resistance, attention was turned to small molecule tyrosine kinase inhibitors. Along these lines, lapatinib (small molecular dual tyrosine kinase inhibitor of HER1 and HER2) was evaluated in the setting of recurrent ovarian cancer [33]. When given to patients with epithelial ovarian cancer (EOC) who recurred after frontline chemotherapy, the authors were unable to demonstrate the clinical benefit of lapatinib and topotecan compared to topotecan alone [34, 35]. Unfortunately, in this study, HER2 overexpression was not evaluated/used as eligibility criteria for enrollment. Inspired by the promising data from targeting HER2 in breast cancer with amplification of the *cerbB2* gene [35], continuing efforts to evaluate and establish HER2 as a potential target in gynecologic malignancies were pursued.

Neratinib, an oral, irreversible, tyrosine kinase inhibitor of HER1, HER2, and HER4, has proven efficacy in HER2-positive metastatic breast cancer for both trastuzumab-resistant and trastuzumab-naïve patients, not only in combination with various chemotherapeutic agents [31, 36–38], but also as a single agent [38]. The likely reason that neratinib maintains its efficacy in the presence of trastuzumab resistance is its potent inhibition on HER2 downstream phosphorylation and signaling [31, 39]. Consistent with this view, neratinib has been reported to attain an objective response rate and clinical benefit in 32 and 44% of patients previously treated with anthracyclines, taxanes, and trastuzumab, respectively [37, 40]. Neratinib has been tested as part of both neoadjuvant as well as adjuvant treatment in breast cancer. Namely, the I-SPY 2 trial has reported pCR rate of 30% when neratinib was added to standard neoadjuvant chemotherapy for patients with HER2-positive breast cancer [41]. Given these strong results, neratinib “graduated” from the adaptive I-SPY 2 trial and is eligible to be tested in phase III trials that could potentially lead to its accelerated approval by the FDA [42].

Our group previously reported preclinical activity of neratinib in treatment of uterine serous carcinoma and carcinosarcoma with HER2/neu-amplification, which suggests neratinib as a potentially effective novel agent for patients with HER2/neu overexpressing chemo-resistant gynecologic malignancies [12, 15]. This current paper reports the first preclinical data on neratinib’s efficacy in the treatment of primary ovarian cancer cell lines with HER2/neu-amplification. Although neratinib is a panHER inhibitor, we showed that it is selectively active in HER2-amplified compared to non-amplified ovarian cancer cell lines, which is consistent with previously published preclinical studies in gynecologic as well as non-gynecologic malignancies [12, 15, 43]. Besides its efficacy, neratinib has been reported to be a well tolerated agent with the most common side effect being grade 3 diarrhea [12, 38, 44].

In conclusion, the current study shows significant pre-clinical efficacy of neratinib in the treatment of HER2/neu-amplified, chemoresistant EOC for the first time, by utilizing primary ovarian cancer cell lines. These data from our experiments, combined with clinical data presented above, suggest that neratinib may be a safe and potentially efficacious treatment in ovarian cancer patients resistant to chemotherapy overexpressing HER2/neu.

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Fig. 1.

a Comparison of the mean IC₅₀ values of HER2/neu-amplified versus non-amplified primary epithelial ovarian carcinoma cell lines.

b Comparison of the grouped mean IC₅₀ value for HER2/neu-amplified versus non-amplified cell lines

Fig. 2.

Representative effect of neratinib on tumor cell cycle. Neratinib causes arrest of the cell cycle in G0/G1 with a significant effect seen with both 0.065 and 0.133 µM of drug

Fig. 3.

Representative effect of neratinib on downstream phosphorylation of S6. Tumor cell cycle effects of IC₅₀, half the physiologic dose and the physiologic dose concentrations of neratinib on downstream phosphorylation of the transcription factor S6 at 24 h in HER2/neu-amplified OSPC ARK-1

Fig. 4.
Neratinib treatment improves overall survival in mice with HER2/neu-amplified xenograft
OSPC ARK-1 compared to vehicle

Table 1
Characteristics and demographic data of the six primary ovarian carcinoma cell lines used

Cell line	Age	Race	FIGO stage	Primary site	Histology	IHC cell block	FISH
OSPC ARK-1	69	W	IV	Ovary	Serous	3+	Amplified
OVA(K)56	79	W	IC	Ovary	Clear cell	3+	Amplified
OVA(K)110	51	W	IIC	Ovary	Clear cell	3+	Amplified
CC ARK-2	32	W	IC	Ovary	Clear cell	0	Non-amplified
OSPC ARK-3	53	W	IIIA	Ovary	Serous	0	Non-amplified
OSPC ARK-2	64	W	IIIC	Ovary	Serous	0	Non-amplified

W white, FIGO International Federation of Gynecology and Obstetrics, IHC immunohistochemistry, FISH fluorescent in situ hybridization