

# Triple-negative breast cancer and the potential for targeted therapy

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Breast cancer is composed of several well-recognized subtypes including estrogen receptor, progesterone receptor and HER2 triple-negative breast cancer (TNBC). Without available targeted therapy options, standard of care for TNBC remains chemotherapy. It is of interest to note that TNBC tumors generally have better responses to chemotherapy compared with other subtypes. However, patients without complete response account for approximately 80% of TNBC. Mounting evidence suggests significant heterogeneity within the TNBC subtype, and studies have focused on genetic targets with high rates of altered expression. Recent studies suggest clear possibilities for benefits from targeted therapy in TNBC. In this review, we summarize studies of targeted therapy, including within mouse models, and discuss their applications in the development of combinatorial treatments to treat TNBC.

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The lifetime breast cancer incidence in women is one in eight and it is estimated that 30% of the newly diagnosed women's cancer cases will be breast cancer in 2017. Consistent with mortality trends in all cancers, overall survival rate of breast cancer has increased over the past decade. However, approximately 24% of breast cancer patients are first diagnosed while under 40 years of age and breast cancer is the leading cause of cancer mortality in this age group. In addition, cancer diagnosis prior to 40 is commonly made in African-Americans and *BRCA1/2* mutation carriers [1].

Breast cancer heterogeneity is well known and is commonly observed using routine markers. Clinically, breast cancer is routinely categorized into four subtypes based on the presence of estrogen receptor (ER), progesterone receptor (PR), HER2 and ki67 [2]. With the addition of transcriptomics, many of these ER- and PR-positive tumors have been classified into the commonly observed luminal A and luminal B subtypes [3–5]. Tumors with HER2 amplification and/or overexpression are classified into the HER2-positive subtype. Triple-negative breast cancer (TNBC) represents tumors without the expression of ER, PR and HER2 and largely overlaps with the basal subtype. Although TNBC only represents 15–20% of breast cancer, distant recurrence and mortality in the TNBC is significantly worse than the other subtypes [6]. Considering the malignancy of TNBC and the death rate of metastatic breast cancer, further studies are required to improve the outcome of this subtype of breast cancer.

## Molecular subtypes of breast cancer

With the advent of gene expression microarray technology, seminal manuscripts initially stratified breast cancer into five intrinsic subtypes [3]. These subtypes, including luminal A, luminal B, HER2, basal-like (BL) and normal-like, are differentiated based on gene expression. The BL tumors have high expression of keratin 5/6 and 17; in contrast, the luminal subtype expresses luminal keratin 8/18 [5]. Linked back to clinical markers, 79% of TNBC is defined as BL subtype [7]. With increased samples, inclusion of additional genes and a comparison to mouse models, Perou and colleagues uncovered an additional subtype called claudin-low [8]. Claudin-low tumors are histologically triple negative with increased expression of those genes involved in epithelial-to-mesenchymal transition [9]. Although

these six subtypes have been useful, there is additional complexity within each subtype. Indeed, even within subtypes there is variation in survival as shown when the basal group was subdivided to three unique pathway expression patterns [10]. Lehmann *et al.* categorized TNBC tumors into six subtypes on the basis of the gene expression and gene ontology [11]. The representative gene ontologies in BL1 and BL2 subtypes include cell cycle, EGF and MET pathways. The immunomodulatory subtype was enriched for multiple immune signaling pathways. The mesenchymal-like (M) and mesenchymal stem like (MSL) subtypes have high expression of genes associated with cell differentiation. The luminal androgen receptor (LAR) subtype was marked with increased androgen expression and enriched androgen and estrogen metabolism. Recently, Burstein *et al.* split TNBC patients into four subtypes, LAR, mesenchymal, BL immunosuppressed and BL immune-activated based on mRNA expression and various clustering methods [12]. In an alternative approach using immune metagene information, TNBC tumors were clustered into three subtypes, including LAR, BL with a low-immune response and BL with high-immune response [13]. Given these various classifications based on gene expression, genomic events regulating development of basal breast cancer have been examined, resulting in the identification of deletion of chromosome 5q in many basal tumors [14].

In addition to the deletion event, other heterogeneous characteristics of breast cancer have been noted at the epigenetic and protein expression level [15]. Importantly, these gene expression differences among the subtypes of breast cancer are strongly correlated with the therapeutic responses.

### Molecular characteristics in current treatment & identification of targets

In the past, decisions regarding therapeutic options for patients with different subtypes of breast cancer were based on the histological properties. For instance, hormone therapy is the standard of care for ER- and/or PR-positive breast cancer patients, and HER2-targeted therapy is standard for HER2-positive tumors. Due to the lack of those three receptors in TNBC, chemotherapy is still the primary option for these patients. Although TNBC is the subtype with the most complete response to chemotherapy (22%), the recurrence and metastasis rate of TNBC patients was also higher than non-TNBC tumors [16]. The urgent need to improve the therapeutic outcomes of TNBC patients is the focus of numerous studies on the association of molecular characteristics with this subtype, therapeutic responses and identification of new targets.

### Molecular characteristics in response to chemotherapy

In addition to histological subtypes directing therapeutic options, a number of studies have worked on linking the correlation between molecular characteristics of subtypes of breast cancer and their therapeutic outcomes. For example, a recent report outlined that BL and HER2+ve patients have a better response to taxane and anthracycline treatment. Interestingly, the gene expression patterns associated with therapeutic responses in these two populations were distinct [17]. Among the basal patients, half of BL1 patients had pathological complete responses while BL2 patients had the least treatment benefits from chemotherapy [18]. These studies clearly demonstrate the impact of molecular and genomic heterogeneity in patients receiving the same treatment.

### Molecular characteristics of TNBC & potential targets

To explore the potential targets for TNBC treatment, the genomic impacts inherent within TNBC patients' response to treatment have been examined. Silver *et al.* demonstrated that alteration of BRCA1 expression due to promoter methylation and p53 mutations was correlated with good response to cisplatin treatment [19]. Likewise, high expression of CD73 was related to resistance to doxorubicin in TNBC patients [20]. Another study focusing on the genomic spectrum of BL tumors showed that the genomic alterations in BL tumors include mutations of *p53* and *PIK3CA*, loss of *PTEN* and *RBI*, amplification of *cyclin E1* and increased expression of *Myc* and *HIF1- $\alpha$*  [15]. In addition to the primary tumor, Balko *et al.* analyzed the residual breast cancer after neoadjuvant chemotherapy, and identified additional amplification of several genes (such as *CDK4*, *MCL1*, *JAK2*, *AKT1* and *EGFR*) and loss of mutations in *BRCA1/2*, *ATM* and *CDKN2A* [21]. These findings provide additional information that may well impact development of targeted therapies for TNBC.

Based on the gene expression studies of TNBC and the common characteristics of cancers, several potential targets have been identified in studies from *in vitro*, *in vivo* and clinical trials. A representative summary of current trials of targeted therapy, the combination of different target therapeutic reagents and the combination of target therapy and chemotherapy is listed in Table 1. Here we describe these targets and treatments based on their known

Table 1. Ongoing clinical trials in triple-negative breast cancer.

Target	Drug	Phase	NCT identifiers	Status
<b>BRCA1/2</b>				
PARP	Veliparib	Phase I	NCT00892736	Active, not recruiting
	Veliparib + chemotherapy	Phase I, II or III	NCT02595905; NCT01251874; NCT01145430; NCT00576654; NCT01818063; NCT02032277	Recruiting or active, not recruiting
	Veliparib + lapatinib (EGFR/HER2i)	Pilot study	NCT02158507	Recruiting
	BGB290 + temozolomide	Phase I – Phase II	NCT03150810	Recruiting
	BMN-673 (talazoparib)	Phase II	NCT02401347	Recruiting
	BMN-673 + carboplatin + paclitaxel	Phase I	NCT02358200	Active, not recruiting
	Rucaparib + cisplatin	Phase II	NCT01074970	Active, not recruiting
	Olaparib	Phase II	NCT02681562; NCT00679783	Active, not recruiting
	Olaparib ± carboplatin ± paclitaxel	Phase I	NCT00516724; NCT01445418; NCT00707707	Active, not recruiting
		Phase II; Phase II – Phase III	NCT02789332; NCT03150576	Recruiting
	Olaparib ± AZD2014 (mTORi) ± AZD5363 (AKTi)	Phase I – Phase II	NCT02208375	Recruiting
	Olaparib + onalespib (HSP90i)	Phase I	NCT02898207	Recruiting
	Olaparib ± BKM120 (PI3Ki) ± BYL719 (PI3Ki)	Phase I	NCT01623349	Active, not recruiting
	Olaparib + cediranib (VGEFi)	Phase I – Phase II	NCT01116648	Active, not recruiting
		Phase II	NCT02498613	Recruiting
	<b>Cyclin-dependent kinases</b>			
CDKs	Dinaciclib + epirubicin	Phase I	NCT01624441	Active, not recruiting
	Abemaciclib	Phase II	NCT03130439	Recruiting
	Trilaciclib	Phase II	NCT02978716	Recruiting
	Ribociclib + bicalutamide (ARi)	Phase I – Phase II	NCT03090165	Recruiting
<b>CHK1 and WEE1</b>				
CHK1	LY2880070 + gemcitabine	Phase I – Phase II	NCT02632448	Recruiting
CHK1/2	LY2606368	Phase II	NCT02203513	Recruiting
Wee1	AZD1775 + cisplatin	Phase II	NCT03012477	Recruiting
	AZD1775	Phase I	NCT02482311	Active, not recruiting
<b>Growth factors and angiogenesis</b>				
EGFR	Aftatinib + paclitaxel	Phase II	NCT02511847	Recruiting
	Cetuximab + ixabepilone	Phase II	NCT01097642	Active, not recruiting
	Erlotinib + chemotherapy	Phase II	NCT00491816	Active, not recruiting
	Icotinib	Phase II	NCT02362230	Recruiting
	Panitumumab ± cisplatin ± paclitaxel ± carboplatin	Phase II	NCT02546934; NCT02593175; NCT02876107	Recruiting
HER2	Trastuzumab + paclitaxel + cyclophosphamide	Phase II	NCT01750073	Recruiting
VEGF	Bevacizumab + paclitaxel	Phase II	NCT01898117	Recruiting
	Bevacizumab + paclitaxel + erlotinib (EGFRi)	Phase II	NCT00733408	Active, not recruiting
VEGFR2	Apatinib + vinorelbine	Phase II	NCT03254654	Recruiting
	Apatinib + fluzoparib (PARPi)	Phase I	NCT03075462	Recruiting
VEGFR/FGFR	Lucitanib	Phase II	NCT02202746	Active, not recruiting
<b>Androgen receptor</b>				
Androgen	Enzalutamide	Phase II	NCT02750358	Recruiting
			NCT01889238	Active, not recruiting
	Enzalutamide + paclitaxel	Phase IIb	NCT02689427	Recruiting
	Bicalutamide	Phase III	NCT03055312	Recruiting

The representative ongoing clinical trials of targeted therapy, including recruiting and active, not recruiting ones, are summarized based on the data from ClinicalTrials.gov as of 24 August 2017. The trials of evaluating the effect of the combination of targeted therapy and immunotherapy are excluded. The letter i represents inhibitor.

Table 1. Ongoing clinical trials in triple-negative breast cancer (cont.).

Target	Drug	Phase	NCT identifiers	Status
	GTx-024	Phase II	NCT02368691	Active, not recruiting
CYP17	VT-464	Phase II	NCT02130700	Recruiting
<b>PI3K/AKT/mTOR pathway</b>				
PIK3CA	Taselisib + enzalutamide (ARI)	Phase I – Phase II	NCT02457910	Recruiting
PI3K	BKM120	Phase II	NCT01790932	Active, not recruiting
	BKM120 + MEK162 (MEKi)	Phase I	NCT01363232	Active, not recruiting
	BKM120 + capecitabine + trastuzumab (HER2i)	Phase II	NCT02000882	Recruiting
	AZD8186	Phase I	NCT01884285	Recruiting
	Gedatolisib + docetaxel + cisplatin + dacomitinib (EGFRi)	Phase I	NCT01920061	Recruiting
PI3K/mTOR	PQR309 + eribulin	Phase I – Phase II	NCT02723877	Recruiting
	Tak-228 (mTORi) + Tak-117 (PI3Ki) + cisplatin + paclitaxel	Phase II	NCT03193853	Recruiting
Akt	Ipatasertib	Phase II	NCT02162719; NCT02301988	Active, not recruiting
	ARQ092 + carboplatin + paclitaxel	Phase Ib	NCT02476955	Recruiting
	GSK2141795 + trametinib (MEKi)	Phase II	NCT01964924	Active, not recruiting
mTOR	Everolimus	Phase II	NCT01931163	Recruiting
	Everolimus + eribulin	Phase I	NCT02616848	Recruiting
	Everolimus + cisplatin + paclitaxel	Phase I – Phase II	NCT01031446	Active, not recruiting
		Phase I	NCT02120469	Recruiting
	Everolimus + doxorubicin + bevacizumab (VEGFi)	Phase II	NCT02456857	Recruiting
	Everolimus + cisplatin + paclitaxel	Phase II	NCT02531932	Recruiting
	Everolimus + 5-fluorouracil + epirubicin + cyclophosphamide	Phase II	NCT00499603	Active, not recruiting
<b>Src and WNT signaling</b>				
Src	Dasatinib	Phase II	NCT02720185	Recruiting
WNT	PTK7-ADC (WNTi) + gedatolisib (PI3Ki)	Phase I	NCT03243331	Not yet recruiting

The representative ongoing clinical trials of targeted therapy, including recruiting and active, not recruiting ones, are summarized based on the data from ClinicalTrials.gov as of 24 August 2017. The trials of evaluating the effect of the combination of targeted therapy and immunotherapy are excluded. The letter i represents inhibitor.

functions, including DNA repair and damage, growth and angiogenesis, survival and proliferation for the PI3K pathway and others (Table 2 & Figure 1).

### *DNA damage & repair*

DNA damage responses are important in both normal and cancer cells. It is known that the cytotoxic effects of chemotherapeutic reagents are through various mechanisms. Thus, it is plausible that the alterations of genes involved in the responses to DNA damage could be targets for treating TNBC. In response to DNA damage, CDKs and Chk1 act as checkpoints regulating cell cycle, and BRCA1 is involved in repairing DNA damage.

### **BRCA1 & BRCA2**

A total of 19.5% of TNBC patients have been shown to carry *BRCA1/2* mutations. *BRCA1/2* functions in repairing double-strand breaks through homologous recombination, and PARP mediates nonhomologous end joining and base-excision repair. Inhibition of PARP blocked the alternative DNA repair mechanism mediated by *BRCA1/2* [22]. Mutations of *BRCA1/2* have been shown to sensitize tumor cells to PARP inhibitors, and the

Table 2. Examples of potential targets and drugs for triple-negative breast cancer.

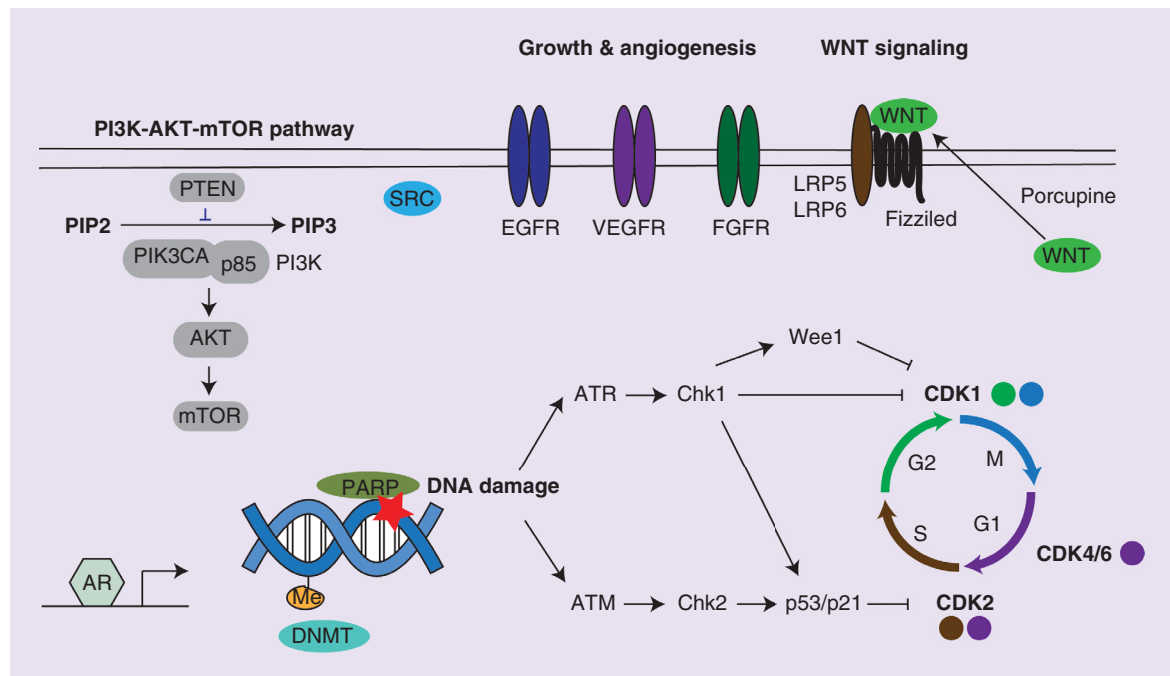
Targets	Drugs	Mechanisms/actions	Ref.
Androgen receptor	Bicalutamide	AR antagonist	[11,46]
	Enzalutamide	AR antagonist	[45]
	17-DMAG	HSP90 inhibitor; regulate the stability of AR	[44]
	VT-464	CYP17 lyase; involved in the synthesis of androgen	[44]
BRCA1/2	Olaparib	Targeting PARP in BRCA1/2 mutant cells	[24]
CDKs	Dinaciclib	Pan-CDK inhibitor; G1/S and G2/M arrest; synthetic lethal in Myc-dependent tumor cells	[28]
	Purvalanol A	CDK1 inhibitor; G2/M arrest; synthetic lethal in Myc-dependent tumor cells	[28]
	RO-3306	CDK1 inhibitor; G2/M arrest; synthetic lethal in Myc-dependent tumor cells	[29]
	CGP74514A	CDK1 inhibitor; G2/M arrest; synthetic lethal in Myc-dependent tumor cells	[29]
	Palbociclib (PD0332991)	CDK4/6 inhibitor; G1/S arrest	[29,30]
	Ribociclib	CDK4/6 inhibitor; G1/S arrest	[27]
CHK1	V158411	Inhibitors sensitize cells to DNA-damaging agents (gemcitabine and cisplatin)	[33]
DNMT1	Decitabine	Inhibit DNA methyltransferase	[58]
EGFR	Cetuximab	EGFR monoclonal antibody	[38]
	Erlotinib	EGFR monoclonal antibody	[39]
FGFR	PD173074	ATP-competitive inhibitor of FGFR	[37]
p53	PRMIA-1	p53 activator; rescue the function of p53 in p53-deficient cells	[36]
	APR-246	p53 activator; rescue the function of p53 in p53-deficient cells	[36]
PI3K/AKT/mTOR	GDC-0941	PI3K inhibitor	[47]
	NVP-BKM120 (BKM-120)	PI3K inhibitor	[47]
	Everolimus	mTOR inhibitor	[48,53]
	Temsirolimus	mTOR inhibitor	[53]
	GDC-0980	PI3K/mTOR dual inhibitor	[47]
	BEZ235 (NVP-BEZ235)	PI3K/mTOR dual inhibitor	[49]
VEGF	Bevacizumab	VEGF monoclonal antibody	[42]
SRC	Dasatinib	SRC inhibitor	[11,55]
Wee1	MK1775	Inhibit Wee1 kinase; G2/M arrest; sensitize cells to cisplatin	[35]
WNT	LGK974	Inhibit a WNT-specific acyltransferase (porcupine) to block the secretion of WNT ligands	[57]

Targeted therapeutic agents and their reported mechanisms and actions are listed.  
AR: Androgen receptor.

combination of a PARP inhibitor (olaparib/AZD2281) and cisplatin or carboplatin prolonged the overall survival of mice implanted with BL mouse tumors [23]. Olaparib has also been tested in clinical trials although resistance has been observed in patients. Patients receiving high doses of olaparib (400 mg twice daily) had a better objective response (41%) with acceptable toxicity compared with those patients treated with low doses of olaparib (100 mg twice daily) [24]. Interestingly, a second mutation of *BRCA1* can restore the normal functions of *BRCA1* in TNBC patients with acquired resistance to chemotherapy [25].

### Cyclin-dependent kinases

Altered expression of CDKs, including CDK4 and CDK6, has been observed in residual TNBC [21]. Cell cycle is tightly regulated by the formation of various cyclin-CDK complexes in normal cells; in contrast, the hyperactive CDK4 and CDK6 expedite G<sub>1</sub>-S transition through phosphorylation of Rb and the release of Rb-bound E2Fs in many cancers. In addition to the G<sub>1</sub> arrest caused by CDK4/6 inhibitors, CDK1/2 inhibition results in cell cycle arrest and apoptosis [26]. Recently, an aromatase inhibitor plus ribociclib (a CDK4/6 inhibitor) has been approved for hormone-receptor-positive/HER2-negative metastatic breast cancer after successful clinical trials [27]. Preliminary *in vitro* and *in vivo* studies illustrated the potential of targeting CDKs in TNBC. Inhibition of CDKs using a pan-CDK inhibitor (dinaciclib) or a CDK1 inhibitor (purvalanol A) had a synthetic lethal effect in TNBC cells with elevated Myc pathway activity compared with receptor-positive breast cancer cell lines [28]. Later, another study concluded that Myc-dependent breast cancer cells are more sensitive to CDK1 inhibitors compared with



**Figure 1. Potential targets for triple-negative breast cancer.** A simplified schematic figure of all potential targets discussed is depicted. The colored circles of CDKs represent the corresponding phases of the cell cycle. AR: Androgen receptor; ATM: Ataxia-telangiectasia mutated; ATR: Ataxia telangiectasia and Rad3 related; DNMT: DNA methyltransferase; Me: Methyl group.

other CDK inhibitors [29]. In addition, inhibition of CDK4 decreased the percentage of chemotherapy-resistant cells and breast cancer stem cells in TNBC [30]. However, the subtype-specific benefit of CDK inhibitors has not yet been elucidated in TNBC patients.

### P53 & CHK1

Mutations in p53 have been noted in more than 80% of BL breast cancers [15]. This is a significant opportunity for therapy in TNBC. In response to double-strand DNA breaks, the ATM-Chk2 pathway is activated, followed by the activation of p53. The ATR kinases bind to the complex at the single-stranded DNA sites, and subsequently activate Chk1 [31]. Thus, Chk1 serves as a druggable target in p53-deficient tumor cells. Indeed, inhibiting Chk1 sensitized p53-mutant TNBC cells to DNA damaging agents [32]. However, Chk1 inhibition in patients remains an open area of investigation due to the toxicity observed in clinical trials of other cancers [33]. Instead of targeting Chk1 directly, an emerging approach is to inhibit the downstream of Cdk1, WEE1. The activated Wee1 phosphorylates CDK1 and causes G<sub>2</sub>/M arrest [34]. The combination of MK1775 (a Wee1 inhibitor) and cisplatin resulted in enhanced tumor growth inhibition in a TNBC xenograft model (MDA-MB-231) [35]. Furthermore, in a promising line of investigation, Synnott *et al.* have shown that two compounds (PRMIA-1 and APR-246) can rescue p53 functions in p53-deficient TNBC cells and had synergistic therapeutic effects when combined with chemotherapy [36].

### Growth factors & angiogenesis

EGFR, FGFRs and VEGFRs are major growth factors consistently found overexpressed or amplified in TNBC patients [15,21,37]. Upon ligand binding, these growth factors activate the downstream signaling promoting tumor growth and angiogenesis. The EGFR inhibitors (antibodies and tyrosine kinase inhibitors) have been routinely used in the clinic to treat non-small-cell lung cancer. Addition of cetuximab (an anti-EGFR antibody) to cisplatin significantly increased the response rate (overall survival: 20 vs 10%, and progression-free survival: 3.7 vs 1.5 months) in metastatic TNBC patients [38]. However, in another Phase I clinical trial of erlotinib (an EGFR inhibitor) plus bendamustine (a nitrogen mustard derivative alkylating agent) in metastatic TNBC patients, severe lymphopenia was noted and only one out of 11 patients had partial responses [39] (ClinicalTrials.gov number NCT00834678). No selective FGF inhibitors have been approved by US FDA in cancer treatment, and no clinical trials of the FGF



inhibitors have been completed in TNBC. Preliminary studies with PD173074 (an FGFR inhibitor) inhibited the growth of TNBC cells known with amplified FGFR2 and CAL-51 (a BL breast cancer cell line) xenografts [37,40]. The indications of bevacizumab (a VEGFR inhibitor) include treatments in non-small-cell lung cancer, metastatic colorectal cancer and other cancers. However, the combination of bevacizumab and chemotherapy in HER2-ve metastatic breast cancer did not improve overall survival significantly [41]. Another completed Phase III clinical trial reported that the addition of bevacizumab to chemotherapy did not prolong the overall survival or invasive disease-free survival, indicating that further studies are required to define the benefits of the combination of bevacizumab and chemotherapy in TNBC patients [42].

### Androgen receptor

The expression of androgen receptor (AR) has been noted in 60.5% of breast cancer patients, correlating with increased overall survival and disease-free survival [43]. However, in the LAR subtype of TNBC, elevated AR expression was associated with decreased relapse-free survival [11]. In addition to androgen receptor, examples of known alternative targets are HSP90 which regulates the function and the stability of ARs and CYP17 lyase which is required in the synthesis of androgen [44]. Four human breast cancer cell lines sharing gene expression patterns with LAR TNBC had higher sensitivity to bicalutamide (AR antagonist) or 17-DMAG (a HSP90 inhibitor) than those ones shared with BL TNBC [11]. Cochrane *et al.* concluded that enzalutamide was effective in treating AR-positive TNBC cells or ER+ cells *in vitro* and in xenografts [45]. A Phase II clinical trial of bicalutamide (150 mg daily) in AR+ve/PR-ve/ER-ve metastatic breast cancer patients showed that 19% of patients have a therapeutic response to this agent for at least 6 months, and the toxicity of this treatment is moderate [46]. In addition to those AR inhibitors, a clinical trial will evaluate the benefit of a CYP17 lyase inhibitor in AR+ve TNBC patients (ClinicalTrials.gov number NCT02130700).

### PI3K/AKT/mTOR pathway

PIK3CA mutation frequency was noted to be elevated in both luminal and HER2 subtype tumors, ranging from 32 to 49%. In contrast, basal subtype tumors are enriched for loss of PTEN and amplification of AKT3 [15]. In TNBC, the PIK3CA mutation rate was significantly increased in the residual TNBC and AR-positive TNBC [11,21]. Given these findings, it is interesting to note that a PI3K inhibitor plus bicalutamide synergistically inhibited the growth of AR-positive TNBC cells [47]. Everolimus (an mTOR inhibitor) plus exemestane (an aromatase inhibitor) extended the progression-free survival of metastatic hormone-receptor-positive breast cancer [48], and has been approved for clinical use. Translating this to TNBC, activation of mTOR was observed in TNBC, and BEZ235 (a PI3K/mTOR inhibitor) enhanced the tumor inhibition effects of chemotherapy reagents (carboplatin and docetaxel) in mice orthotopically injected with MDA-MB231 cells in a proof of concept experiment [49]. In addition, clinical trials have evaluated the clinical benefits of combinations of chemotherapy and PI3K or mTOR inhibitors in TNBC. A Phase II trial of GDC-0941 (a PI3K inhibitor) plus cisplatin in metastatic TNBC was terminated (ClinicalTrials.gov number NCT01918306). Another Phase II trial showed that 36% of metastatic TNBC patients benefited from the combination of everolimus and carboplatin [50] (ClinicalTrials.gov number NCT01127763). Compared with the arm receiving chemotherapy, the combined everolimus and chemotherapy did not improve the complete response. Of note, the mutations in PI3K signaling were not related to the response to the treatment [51] (ClinicalTrials.gov number NCT00930930).

Additionally, several feedback loops involved in the resistance to the inhibitors targeting PI3K/mTOR pathway have been identified [52]. Some trials have focused on the combination of different targeted therapeutic agents. For instance, a Phase I clinical trial has evaluated the combination of BEZ235 and MEK162 (a MEK1/2 inhibitor) in TNBC and other cancers (completed but results not yet available; ClinicalTrials.gov number NCT01337765). The combination of doxorubicin, bevacizumab and mTOR inhibitors (temsirolimus or everolimus) has been used in a Phase I clinical trial in metaplastic TNBC. Interestingly, this study found that patients with abnormal PI3K activation have a better objective response rate [53]. Taken together, these studies suggest that further analysis is required for determining the subpopulation of TNBC which will benefit from PI3K/mTOR inhibitors treatment.

### SRC, WNT signaling & DNMT1

Compared with non-TNBC tumors, detection of Src at the membrane was significantly increased in TNBC tumors [54]. Considered with this finding, it is not surprising that TNBC cell lines are more sensitive to the Src inhibitor (dasatinib), especially in the M and MSL subtypes [11]. However, only 4.7% of TNBC patients had

complete or partial response to dasatinib treatment in a Phase II study [55]. In addition to Src, WNT signaling was enhanced in BL2, M and MSL subtypes of TNBC [11], and was correlated with development of lung and brain metastases [56]. LGK974 (a WNT-specific O-acyltransferase porcupine inhibitor) inhibited WNT signaling in a mouse model of breast cancer [57], and has been proposed in a clinical trial in TNBC and other cancers (ClinicalTrials.gov number, NCT01351103). An additional gene elevated in TNBC is *DNMT1*, playing a key role in maintenance of methylation. At the expression level, DNMT1 was elevated in TNBC [58]. The combination of the DNMT inhibitor (decitabine) and PARP inhibitors resulted in additional tumor inhibition in wild-type BRCA1 TNBC cells [59]. Currently, 17 early-stage clinical trials targeting WNT signaling in cancers other than breast cancer are underway or are recruiting patients (based on data from ClinicalTrials.gov as of 22 August 2017). Conversely, both DNMT inhibitors and Src inhibitors have been approved by FDA for treating other cancers, such as myeloid malignancies.

### Development of new targeted therapies & beyond

With the recent advances in 'omics', the number of novel targets has continued to rise for potential pathways that could be used to treat TNBC. This includes several new approaches described below to identify therapy for previously undruggable targets. Given the heterogeneity of TNBC, the combination of these varied methods will be refined to treat specific patients.

### Exploiting 'omics' to identify new therapeutic targets & treatments

In recent years, genome- and transcriptome-wide studies have been the mainstay of identifying genes associated with the sensitivity and resistance to chemotherapy in TNBC as well as the BL subtype. For instance, tumors with genetic aberrations may be vulnerable to specific treatments based on specific expression or pathway effects. Likewise, more recent studies in epigenomic and proteomic analyses of TNBC have delineated therapeutic potential of various targets. With the development of these various sources of 'omic' data in hand, drug repurposing is now feasible. This essentially integrates the expression of various genetic programs with drug response data through approaches such as Connectivity Map [60]. However, to date relatively few drugs have been identified this way for breast cancer treatment [61,62]. With additional data and stratification of tumor types, there is obvious potential in this approach. The various means to use 'omic' data are reviewed below.

#### *Sensitivity & resistance*

The relevance of several novel targets to chemotherapy resistance has recently been investigated. For example, CD73 is highly expressed in TNBC patients and is related to the resistance to anthracycline treatment. Mechanistically, CD73 activates A2A adenosine receptor to inhibit CD8<sup>+</sup> cells. The combination of the A2A antagonist and doxorubicin inhibited tumor growth in mice more efficiently than doxorubicin alone [20]. A2A antagonists also decreased the number of lung metastasis colonies in mice injected with CD73<sup>+</sup> mouse mammary tumor cells significantly [63]. Likewise, overexpression of IRAK1 in TNBC patients was associated with resistance to paclitaxel, and IRAK-inh (the IRAK1 inhibitor) rescued the sensitivity to paclitaxel in paclitaxel-resistant cells. Increased expression of IRAK1 was found both in metastatic TNBC cells and patients with poorly differentiated breast tumors. IRAK-inh also reduced the lung metastasis in mice engrafted with metastatic breast cancer cells [64]. In addition to the role of resensitizing cells to chemotherapy, these two emerging targets also target metastasis.

#### *Vulnerability*

In addition to the studies of sensitivity and resistance caused by the alteration of single genes, recent omic studies highlight two functional dependencies within TNBC. First, BL TNBC cells with increased expression of genes involved in proteasome degradation had a markedly better response to proteasome inhibitors relative to other subtypes [65]. In a good example of drug repurposing targeting the protein translation pathway, a recent study identified that compared with Rb1-mutated cells, TNBC cells with deficient Rb1 and p53 were more sensitive to the mitochondrial protein translation pathway inhibitor tigecycline, an FDA-approved antibiotic [61]. In a second line of investigation, elevated synthesis of pyrimidines was observed in TNBC cells after chemotherapy. After blocking *de novo* pyrimidine synthesis, the sensitivity of TNBC cells to chemotherapeutic agents was restored [66]. These approaches demonstrate the utility of targeting broad functional pathways instead of individual genes to restore vulnerability to chemotherapy in TNBC.



### Undruggable targets

There are several genetic pathways elevated in numerous cancers, including both Myc and Ras, that lack targeted therapy and these molecules have been termed ‘undruggable’. Despite the difficulty in directly inhibiting these pathways, their expression and importance in cancer have ensured the continued exploration of methods to target these pathways indirectly. Myc is of particular interest since a large number of TNBC cases have Myc overexpression. Interestingly, a recent metabolomic study identified a synthetic lethal approach for TNBC, including those with Myc overexpression. In this study, using an unbiased screening approach in TNBC, LDHB was found to contribute to the glycolysis dependency of TNBC cells, and knockdown of LDHB inhibited tumor growth in xenograft mice. Considering that the expression of LDHB in TNBC was higher than that in luminal subtypes and high expression of LDHB was correlated with survival, LDHB was noted as potentially having a role in the treatment of TNBC [67]. Another study examined the depletion of essential genes in the fatty acid oxidation pathway which blocked the growth of TNBC cells with high Myc expression *in vitro* and *in vivo* [68]. In addition to these broad pathways, patients with elevated Myc expression were also noted to have elevated SHP2 and ZEB1, correlating with poor prognosis. Moreover, TNBC expression of ERK1, ERK2 and ZEB1 was activated by SHP2 [69]. Thus, when SHP2 was inhibited in TNBC cells, this resulted in decreased activity of multiple signaling pathways, including EGFR, Ras and Akt [70]. Together these examples demonstrate the opportunities to use alternative approaches to target undruggable pathways.

### Combination of therapeutic targets to combat TNBC

The concept of combining different therapeutic agents for synergistic benefits in treatment has been utilized for decades. Moving forward, this may be especially valuable in TNBC where personalized medicine targeting signaling molecules is difficult. In TNBC, as the number of synthetic lethal treatments, identification of novel targets and methods for combatting drug resistance increase and are better understood, it will be critical to assess potential for combinatorial treatment. Driving this forward are studies reviewed below with transgenic mice, human TNBC cell lines, xenografts with these cell lines and patient-derived xenografts (PDXs). These various tools provide a key opportunity to elucidate the response of heterogeneous TNBC to various combination therapies in different experimental models prior to clinical trials. The results of this work can be compared with addition of targeted therapy to chemotherapy in TNBC patients, with several studies reporting remarkable improvements in the response rate in clinical trials.

### Targets associated with response to treatment

A major strategy in improving TNBC treatment outcomes is to combine targets that mediate the responses to current chemotherapy, including those genes identified from the omic studies mentioned previously. One method that has added significantly to this data is high-throughput screening in cell lines to a collection of drugs, revealing both synthetic lethal interactions and identifying genes conferring resistance to targeted therapy. Indeed, in addition to confirming established interactions, this approach revealed that NOTCH1 pathway activation resulted in a surprising resistance to PI3K inhibitors [71]. Given the importance of PI3K inhibitors in breast cancer, and the frequent resistance to PI3K inhibitors, this work is highly significant. Recent work by Castel *et al.* was significant in identifying a new mechanism of PI3K resistance, mediated by PDK1 and SGK1 [72]. Indeed, with PI3K inhibition and development of resistance, they noted SGK1-mediated mTORC1 activity. Addition of a PDK1 inhibitor then rescued the sensitivity of the PI3K inhibitor in these cell lines [72]. Another example of target upregulation with inhibitor treatment was observed with MEK inhibitors. In TNBC cells treated with an MEK inhibitor, elevated activation of several receptor tyrosine kinases, such as AXL, VEGFR2 and PDGFR- $\beta$ , has been noted. The combination of AZD6244 (an MEK inhibitor) and sorafenib (a multiple receptor kinase inhibitor) led to a synergistic inhibition of tumor growth [73].

### Targeting cancers with shared mutations

In TNBC and ovarian cancer, *BRCA1/2* mutations are commonly observed. However, the development of a second mutation in *BRCA1* is associated with resistance to chemotherapy in both ovarian and breast cancers [25,74]. Moreover, mutations of *CDK12* often co-occur with *BRCA1/2* mutations. In two studies, the depletion of *CDK12* expression rescued the sensitivity to PARP inhibitors in ovarian cancer and in TNBC cell lines [75,76]. Following this, a preclinical study of the combination of PI3K and PARP inhibitors demonstrated the synergistic effects in these two cancers. A recent Phase I clinical trial strongly suggests that this inhibitor combination is practical in

treating both cancers [77]. Together, these studies demonstrate the utility of targeting mutations that co-occur in TNBC.

#### *Signaling pathway & network-based approaches*

Gene expression data from breast cancer can be analyzed through the previously described intrinsic approach to stratify into various subtypes. However, this does not allow for the identification of signaling pathway activity. By using gene set enrichment analysis [78] or a Bayesian approach to predict cell signaling pathway activation, considerable knowledge as to the active signaling components within a tumor can be gained [79–81]. Indeed, by combining these predictions with known inhibitors there is significant potential to predict the optimal treatment. For instance, it has been demonstrated that each subtype of breast cancer is enriched for specific pathways while maintaining significant heterogeneity for these pathways [10]. This may well explain the variability in response when individual tumors within a subtype have differential responses to the same drugs. Moving forward in therapeutic development using this type of data, initial studies have focused on overcoming the resistance to one targeted therapeutic agent by adding another one. For instance, the resistance to PI3K/Akt/mTOR pathway in TNBC was addressed by combining these pathway inhibitors with an inhibitor of JAK2 (NVP-BSK805) [82]. However, our recent publication has combined the strategies of developing treatments based on computational analysis of pathways with a combination therapy protocol. In this report we described the development of tailored combinatorial treatments for heterogeneous subtypes of mammary tumors after analyzing the pathway activation patterns, and observed a significant inhibition of tumor growth [83]. Importantly, while the initial proof of principle work with this study was completed in mouse tumors, we then went on to make and test predictions for human TNBC, demonstrating that a three-drug combination targeting specific cell signaling pathways was able to significantly reduce tumor volume.

#### *Experimental models*

Different models, ranging from cell culture assays to mouse models and PDX samples, have unique advantages in breast cancer research. For instance, cell lines are routinely used to understand the characteristics and mechanisms inherent in breast cancer. Advantage of cell line research is that it permits high-throughput drug or RNA interference screening to rapidly develop information about drug resistance or susceptibility. However, given the additional mutations accumulated over time in culture, there may be limitations for the ability to extend tissue culture findings to breast cancer. To address this, one additional avenue of research has been the use of mouse models. Various models, ranging from transgenics to knockouts and chemical mutagens have been used to study many facets of breast cancer, including tumorigenesis, tumor microenvironment and metastasis. Of note, decoding the genomic information from tumors in transgenic mice has demonstrated the similarity between transgenic mouse models of breast cancer and human breast cancer. For instance, significant gene-expression heterogeneity has been observed in both *MMTV-Myc* and *MMTV-Met* transgenic mice, with noted similarity to human breast cancer [84–86]. Moving past gene expression, the same data have been used to predict copy number alterations across numerous transgenic mouse models, revealing potential driver events shared between mouse and human breast cancer [87]. In addition to the basic characterization of the mouse models, recent studies have shown a promising role for transgenic mice in development of treatment. Drost *et al.* found that the transgenic mice carrying different *BRCA1* mutations have disparate responses to targeted therapy, and went on to identify a domain of the *BRCA1* protein essential for the drug resistance [88]. For those targets without FDA-approved drugs, ablation of targets in transgenic mice is another method to examine the widespread effects of targets in a certain oncogene-driven background, such as knockout *Stat3* in *Myc* mice [89]. A more recent advance has been the development of PDX models, particularly in TNBC research. These PDXs recapitulate the molecular characteristics of both primary and metastatic breast cancer [90]. In one study of *BRCA1*-associated drug resistance, one PDX sample had intrinsic resistance to PARP inhibitors, similar to the original breast cancer patient [76]. More importantly, a platform with collections of PDX samples at multiple institutes will be highly influential in examining the response to drug treatments [91].

#### **Future perspective**

The focus of understanding TNBC within the past several years has led to significant progress in the development of targeted therapy and combinatorial treatments. This is exemplified in one of the newest findings in the I-SPY2 trial where the promising benefit of using PARP inhibitor contained treatment in unselected TNBC patients was

observed [92]. In this review, we have described some of the major approaches used to address the unmet needs for TNBC treatments. Here, we discuss a couple of challenges that remain in TNBC treatment.

First, a major challenge that remains is both inter- and intratumor heterogeneity. To address this, TNBC patients should be stratified into subtypes based on the integrated omics data. Moreover, the examination of intratumor heterogeneity is required for the development of treatments. As one tumor consists of multiple colonies, these colonies may well have diverse functions and orchestrate the various properties of tumors [93]. In addition, if one dominant clone is targeted, other metastatic clones or other minor clones within the tumor may continue to grow and metastasize.

Indeed, the advances in single-cell sequencing have enabled the interpretation of the differential gene expression among the populations of cells in a tumor, and the ability to trace the evolution of TNBC [94]. This indicates the requirement of a deeper and broader understanding of targets and targeted therapeutic agents, especially for rare populations, including cancer stem cells and metastatic cells.

A second major hurdle is to uncover additional drivers, modifying genes and potential targets for development of effective therapy for TNBC. With each new screen, it is clear that only a portion of the targets involved in key networks have been uncovered. In order to maximize effectiveness of combinatorial therapy, these events and their mechanistic details need to be elucidated.

A third challenge is the integration of omic technologies with the various model systems. Although many investigators are expert in mouse models, PDX, drug screening or omic technologies, it is still rare for these disciplines to be combined. Given that the NCI's Molecular Analysis for Therapy Choice (MATCH) trial pan-cancer Phase II trial integrates alterations in gene expression in tumors with determination for treatment modalities, it is clear that this type of approach is required [95]. It is anticipated that the MATCH trial will accelerate approval of targeted therapeutic agents in different cancers with the same gene alterations. Extending this approach to mouse models, PDX samples with mechanistic understanding will open up new treatment regimens, especially for patients with resistant tumors.

#### Executive summary

##### Molecular subtypes of breast cancer

- Different classifications of subtypes of breast cancer.
- Triple-negative breast cancer (TNBC) has been stratified into at least three subtypes.

##### Molecular characteristics in current treatment & identification of targets

- Subtypes of breast cancer or TNBC have differential responses to the same treatment.
- Highlight targets chosen mainly based on the prevalence of aberrations.
- Most of these targets have been widely studied *in vitro*, *in vivo* and clinical trials.
- BRCA1/2-deficient patients are sensitive to PARP inhibitors.
- CDK4/6 inhibitors have been approved in the hormone-receptor-positive metastatic breast cancer treatment.
- Targets used in treating other cancers, such as SRC, androgen receptor and DNMT1.
- Examples of targets associated with the malignant characteristics.
- Other targets include PI3K/mTOR, p53, CHK1 and WNT signaling.

##### Development of new targeted therapy & beyond

- A list of targets has been identified based on the omics approaches.
- Targeting genes associated with sensitivity and resistance to chemotherapy or targeted therapy.
- Genetic alterations make tumors vulnerable to targeted therapy.
- Some of the novel targets in cancer treatment were identified from studies of metabolomics and epigenetics.
- Overcome the resistances to current treatments through combining targeted therapy agents.
- Extend the application of the same combinatorial treatments for other types of cancer to TNBC based on the shared alterations.
- Signaling pathway based combinatorial treatments led to the inhibition of subtypes of breast cancer individually.
- Recent advances in mouse models offer opportunities to assess the treatments in preclinical stages.

##### Future perspective

- Inter- and intratumor heterogeneity are still challenges.
- Uncovering the additional drivers to treat TNBC systematically and efficiently.
- Integrative approaches are critical in the development of TNBC treatment.

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