Patterns of Mutation Enrichment in Metastatic Triple-Negative Breast Cancer

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ABSTRACT

BACKGROUND: Triple-negative breast cancer (TNBC) is a heterogeneous disease with aggressive biology and complex tumor evolution. Our purpose was to identify enrichment patterns of genomic alterations in metastatic triple-negative breast cancer (mTNBC).

METHODS: Genomic data were retrieved (mutations and copy number variations) from 550 primary TNBC tumors from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) data sets and 58 mTNBC tumors from "Mutational Profile of Metastatic Breast Cancers" and "The Metastatic Breast Cancer Project." Statistical analysis of microarray data between primary and metastatic tumors was performed using a chi-square test, and the percentage of mutation enrichment in mTNBC cases was estimated. *P*-values were adjusted for multiple testing with Benjamini-Hochberg method with a false-discovery rate (FDR) <.05. In addition, we identified dominant hallmarks of cancer in mTNBC.

RESULTS: Seven genes with mutations were enriched in mTNBC after correcting for multiple testing. These included *TTN, HMCN1, RELN, PKHD1L1, DMD, FRAS1*, and *RYR3*. Only RPS6KB2 amplification was statistically significant in mTNBC; on the contrary, deletion of the genes *TET1, RHOA, EPHA5, SET, KCNJ5, ABCG4, NKX3-1, SDHB, IGF2*, and *BRCA1* were the most frequent. The molecular alterations related to the hallmark of "genetic instability and mutation" were predominant in mTNBC. Interestingly, the hallmark of "activating immune destruction" was the least represented in mTNBC.

CONCLUSION: Despite the study limitations, we identified recurrent patterns of genomic alterations with potential contribution to tumor evolution. Deletions were the aberrations more commonly found in mTNBC. Several molecular alterations are potentially targetable.

KEYWORDS: Triple-negative breast cancer, hallmarks of cancer, biomarker, copy number variation, mutation, amplification, deletion

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Introduction

Triple-negative breast cancer (TNBC) corresponds to a group of heterogeneous and highly aggressive tumors characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and HER2.[1] Despite the biological aggressiveness, these tumors present up to 30% of pathological complete responses after neoadjuvant chemotherapy, conferring good prognosis to patients, while those cases with residual diseases have the worse outcomes.[2]

Triple-negative breast cancers can be classified based on their gene-expression profiling in six distinct subtypes: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and a luminal androgen receptor (LAR) subtype;[3] however, the subtypes IM and MSL have an mRNA background from immune and stromal cells, respectively, redefining the TNBC subtypes to only four groups: BL1, BL2, M, and LAR.[4]

In a recent study, Bareche et al, reported that the TNBC subtypes have diverse molecular patterns with substantial differences in activating the hallmarks of cancer, while the BL1 subtype is genetically unstable, LAR tumors have a higher mutational burden. Mesenchymal and MSL subtypes have an increased angiogenesis, and IM present higher expression of immune checkpoint genes.[5]

Triple-negative breast cancer has an intriguing molecular biology. These tumors present ≈80% of mutations in *TP53*, in sharp contrast to the luminal subtype (10%-30%); one consequence of the aforementioned is the high genomic instability in TNBC associated with a wide and continuous spectrum of clonal evolution distinct to other breast cancer subtypes.[6,7] Single-cell sequencing studies showed the patterns of clonal evolution of TNBC, where preexisting resistant cells undergo natural selection after chemotherapy accompanied by transcriptional reprogramming.[8] In this way, copy number variations (CNVs) seem to be an early event during tumor evolution, present even before chemotherapy exposition.[9]

We conducted this study to identify patterns of enrichment of gene alterations through metastases in TNBC and then evaluate how these changes activate specific hallmarks of cancer during tumor evolution.

Table 1. Mutations enriched in mTNBC.

GENE	PTNBC (%)	MTNBC (%)	ENRICHMENT	*P*-VALUE	ADJUSTED *P*-VALUE
TTN	6.5	31.0	374.1	.0000000005	.0000000675
HMCN1	1.1	8.6	690.2	.0000427489	.0028855508
RELN	1.1	8.6	690.2	.0000427489	.0019237005
PKHD1L1	1.6	10.3	532.2	.0000477342	.0016110293
DMD	1.1	6.9	532.2	.0009452504	.0255217608
FRAS1	1.3	6.9	441.9	.0022409009	.0432173745
RYR3	1.3	6.9	441.9	.0022409009	.0378152027

Abbreviations: pTNBC, primary triple-negative breast cancer; mTNBC, metastatic triple-negative breast cancer.

Methods

Data sets for primary and metastatic tumors

We retrieved genomic data regarding mutations and CNV of 550 primary triple-negative breast cancer (pTNBC) tumors from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) data sets and 58 metastatic triple-negative breast cancer (mTNBC) tumors from the "Mutational Profile of Metastatic Breast Cancers" and "The Metastatic Breast Cancer Project." The genomic data of the cohorts was retrieved from http://www.cbioportal.org/.

Identification of enriched genes in metastatic tumors

Differences in proportions of alterations (mutations and CNV) between primary versus metastatic tumors were evaluated by chi-square test, and the percentage of enrichment of alteration in each gene was estimated using the following formula

$$\text{Enrichment} = \frac{\%\text{ in mTNBC} - \%\text{ in pTNBC}}{\%\text{ in pTNBC}} \times 100$$

P-values were adjusted for multiple testing with Benjamini-Hochberg method considering a false-discovery rate (FDR) $<.05$.

Analysis of hallmarks of cancer

The influence of altered genes enriched in metastatic conditions in the hallmarks of cancer was analyzed through normalized pointwise mutual information (NPMI) of the Cancer Hallmarks Analytics Tool (CHAT).[10] The analysis was conducted on an online platform (http://chat.lionproject.net/).

Protein-protein interaction network

We conducted an analysis for protein-protein interaction of the altered genes using the online platform STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, version 10.5, STRING Consortium©) available at http://string-db.org. The analysis was performed with a medium confidence level (0.4). A green line indicates activation; red line, inhibition; blue line, binding; pink line, posttranslational modifications; and yellow line, expression.

Results

Mutations enriched in mTNBC

In total, 135 mutations were present in both primary and metastatic tumors, and 20 genes had a *P*-value $<.05$ in the analysis using chi-square test. After adjusting for multiple comparisons, only mutations in seven genes were statistically significant in mTNBC. These genes included *TTN, HMCN1, RELN, PKHD1L1, DMD, FRAS1*, and *RYR3* (Table 1).

Copy number variations enriched in mTNBC

In total, 661 amplifications were present in both, the primary and metastatic TNBC. Chi-square test identified 17 genes statistically different between the two groups. Only *RPS6KB2* was significantly enriched after adjusting for multiple comparisons. On the contrary, 344 genes with deletions were identified, and from them, 94 presented relevant differences between primary and metastatic tumors. Finally, after adjusting for multiple comparisons, 76 deletions were significant in mTNBC including *TET1, RHOA, EPHA5, SET, KCNJ5, NKX3-1, ABCG4, SDHB, IGF2, BRCA1, SESN3, PMS2, CSF1R, CD74, TSC1, SKP2, PLCG2, PIK3C2G, EIF4A2, ZNF331, FNBP1, ASXL3, PDGFRB, U2AF2, PBRM1, KMT2A, EPHB3, IL7R, DKK1, NUP214, SETD2, DDX6, NAV3, RPS14, ETV5, ABL1, MAP3K13, FLT4, FANCM, TOP1, TFPT, SLC34A2, FLI1, ETS1, NUP98, SPEN, CARS, EPHA2, MKI67, DNAH12, ARHGEF12, TEK, PRDM2, EPHA8, CDC42, CBL, DDX10, TICAM1, KDM4C, MEF2A, NCKIPSD, ZBTB16, EPHB2, FZD10, CHEK1, PAX7, ATM, RICTOR, AKT1, EGFR, AHNAK2, ZNF300, ACVR1B, IGF1R, PHOX2B*, and *LRP1B* (Table 2).

Table 2. Copy number variations enriched in TNBC.

GENE	PTNBC (%)	MTNBC (%)	ENRICHMENT	*P*-VALUE	ADJUSTED *P*-VALUE
Amplifications					
RPS6KB2	1.09	8.62	690.2299	4.2749E−05	.02817153
Deletions					
TET1	0.50	8.60	1480.5	2.85E−07	9.8031E-05
RHOA	0.40	6.90	1796.6	1.6914E−06	.00029091
SET	0.20	5.20	2744.8	7.7681E−06	.00066806
EPHA5	0.20	5.20	2744.8	7.7681E−06	.00089074
SDHB	0.40	5.20	1322.4	.00011484	.00493791
ABCG4	0.40	5.20	1322.4	.00011484	.00564332
KCNJ5	0.70	6.90	848.3	8.7971E−05	.00605238
TFPT	0.20	3.40	1796.6	.00073375	.00615632
TOP1	0.20	3.40	1796.6	.00073375	.00631023
FANCM	0.20	3.40	1796.6	.00073375	.00647203
NKX3-1	0.40	5.20	1322.4	.00011484	.00658387
FLT4	0.20	3.40	1796.6	.00073375	.00664235
MAP3K13	0.20	3.40	1796.6	.00073375	.00682187
ABL1	0.20	3.40	1796.6	.00073375	.00701137
ETV5	0.20	3.40	1796.6	.00073375	.00721169
RPS14	0.20	3.40	1796.6	.00073375	.0074238
NAV3	0.20	3.40	1796.6	.00073375	.00764877
DDX6	0.20	3.40	1796.6	.00073375	.00788779
SETD2	0.20	3.40	1796.6	.00073375	.00814224
NUP214	0.20	3.40	1796.6	.00073375	.00841364
DKK1	0.20	3.40	1796.6	.00073375	.00870377
IL7R	0.20	3.40	1796.6	.00073375	.00901462
EPHB3	0.20	3.40	1796.6	.00073375	.00934849
KMT2A	0.20	3.40	1796.6	.00073375	.00970805
PBRM1	0.20	3.40	1796.6	.00073375	.01009637
U2AF2	0.20	3.40	1796.6	.00073375	.01051705
PDGFRB	0.20	3.40	1796.6	.00073375	.01097432
ASXL3	0.20	3.40	1796.6	.00073375	.01147315
FNBP1	0.20	3.40	1796.6	.00073375	.01201949
IGF2	0.90	6.90	658.6	.000329	.01257496
ZNF331	0.20	3.40	1796.6	.00073375	.01262047
EIF4A2	0.20	3.40	1796.6	.00073375	.0132847
PIK3C2G	0.20	3.40	1796.6	.00073375	.01402274
PLCG2	0.20	3.40	1796.6	.00073375	.01484761

(Continued)

Table 2. (Continued)

GENE	PTNBC (%)	MTNBC (%)	ENRICHMENT	*P*-VALUE	ADJUSTED *P*-VALUE
SKP2	0.20	3.40	1796.6	.00073375	.01577558
PRDM2	0.70	5.20	611.2	.0025429	.01650488
TEK	0.70	5.20	611.2	.0025429	.01682228
TSC1	0.20	3.40	1796.6	.00073375	.01682729
ARHGEF12	0.7	5.2	611.2	.0025429030	.0171521300
DNAH12	0.7	5.2	611.2	.0025429030	.0174951726
MKI67	0.7	5.2	611.2	.0025429030	.0178522170
CD74	0.2	3.4	1796.6	.0007337480	.0180292366
EPHA2	0.7	5.2	611.2	.0025429030	.0182241382
SLC34A2	1.3	6.9	441.9	.0022409010	.0183540463
CARS	0.7	5.2	611.2	.0025429030	.0186118858
SPEN	0.7	5.2	611.2	.0025429030	.0190164920
CSF1R	0.2	3.4	1796.6	.0007337480	.0194161009
NUP98	0.7	5.2	611.2	.0025429030	.0194390807
ETS1	0.7	5.2	611.2	.0025429030	.0198808780
FLI1	0.7	5.2	611.2	.0025429030	.0203432240
PMS2	0.2	3.4	1796.6	.0007337480	.0210341093
SESN3	0.2	3.4	1796.6	.0007337480	.0229463011
BRCA1	0.5	5.2	848.3	.0006975500	.0239957200
IGF1R	0.4	3.4	848.3	.0057136480	.0265607421
ACVR1B	0.4	3.4	848.3	.0057136480	.0269245878
ZNF300	0.4	3.4	848.3	.0057136480	.0272985404
AHNAK2	0.4	3.4	848.3	.0057136480	.0276830269
EGFR	0.4	3.4	848.3	.0057136480	.0280784987
AKT1	0.4	3.4	848.3	.0057136480	.0284854335
RICTOR	0.4	3.4	848.3	.0057136480	.0289043369
ATM	0.4	3.4	848.3	.0057136480	.0293357450
PAX7	0.4	3.4	848.3	.0057136480	.0297802259
CHEK1	0.4	3.4	848.3	.0057136480	.0302383833
LRP1B	0.9	5.2	469.0	.0067276310	.0304513824
FZD10	0.4	3.4	848.3	.0057136480	.0307108580
PHOX2B	0.9	5.2	469.0	.0067276310	.0308574009
EPHB2	0.4	3.4	848.3	.0057136480	.0311983319
ZBTB16	0.4	3.4	848.3	.0057136480	.0317015308
NCKIPSD	0.4	3.4	848.3	.0057136480	.0322212281
MEF2A	0.4	3.4	848.3	.0057136480	.0327582485
KDM4C	0.4	3.4	848.3	.0057136480	.0333134731

Table 2. (Continued)

GENE	PTNBC (%)	MTNBC (%)	ENRICHMENT	*P*-VALUE	ADJUSTED *P*-VALUE
TICAM1	0.4	3.4	848.3	.0057136480	.0338878433
DDX10	0.4	3.4	848.3	.0057136480	.0344823669
CBL	0.4	3.4	848.3	.0057136480	.0350981234
CDC42	0.4	3.4	848.3	.0057136480	.0357362711
EPHA8	0.4	3.4	848.3	.0057136480	.0363980539

Abbreviations: pTNBC, primary triple-negative breast cancer; mTNBC, metastatic triple-negative breast cancer.

Influence of alterations in hallmarks of cancer

The analysis of the influence of enriched alterations in mTNBC on the hallmarks of cancer showed that the hallmark "genome instability and mutation" was extensively overrepresented followed by "sustaining proliferative signaling." Paradoxically, the hallmark "avoiding immune destruction" was the least represented (Figure 1).

Protein-protein interaction between genes presenting alterations enriched in mTNBC

We observed that there was an absence of interaction between several genes; however, three clusters were identified. The first group involves genes related to DNA repair, where ATM is the central node. The second group has genes related to cell metabolism, such as *AKT1, RICTOR, TSC1* among others. Tyrosine kinase proteins (receptors and no receptors) were part of the third cluster, containing genes such as *EGFR, PDGFR, IGF1R ABL1* and ephrin (Eph) receptor subfamily (Figure 2).

Discussion

Clonal evolution of TNBC is an intriguing phenomenon where patterns of evolution using single-cell sequencing have been previously unveiled; however, there are several unanswered questions about the key mechanisms favored during metastases.[8,11]

In this work, we found molecular mechanisms that are enriched in the metastatic setting. The main limitation of the study is we evaluated changes in mutational rates between primary versus metastatic cohorts rather analyze a longitudinal cohort. Although single-cell sequencing could be a better approach to evaluate clonal evolution, our approach comprehends genes, hallmarks, and pathways significantly altered in mTNBC.

The evaluation of new activating mutations in cancer is challenging because of the high rate of false-positive results.[12] In our study, mutations in the *TTN* gene (whose germline mutations are associated to familial restrictive cardiomyopathy) were the most frequently mutated gene in mTNBC, although it is commonly described as a false-positive finding. *TTN* encodes for the giant sarcomeric filament Titin.[13] Involvement of *TTN* in cancer remains unclear and several studies suggested it has not a participation in tumorigenesis or cancer progression while other studies suggest a direct involvement in cancer-related pathways.[14-16] *TTN* alterations are more recurrent in TNBC than other breast cancer subtypes.[17]

Regarding *HMCN1*, although its specific function remains unknown, it has been related to cancer cell invasion and metastasis. Interestingly, the intratumor heterogeneity of *HMCN1* is associated with the prognostic of breast cancer.[18] On the contrary, *RELN* is involved in cell migration where a low expression of this gene is associated with poor outcome in breast malignancies.[19,20]

Amplifications were less observed than gene deletions. The *RPS6KB2* gene amplification was enriched in mTNBC. *RPS6KB2* encodes the S6K2 protein, an effector from the mammalian target of rapamycin (mTOR) signaling pathway, promoting protein synthesis and cell proliferation. In altered states, S6K2 produces aberrant mTORC1 function, thus inducing tumorigenesis.[21] A high number of deletions (n = 76) were found in mTNBC. It describes the greater importance of deletions over amplifications. In our analysis, gene deletions were around six-fold higher in mTNBC than in pTNBC (pTNBC: 0.4%-1.3% vs mTNBC: 3.4%-8.6%; Table 2). Our data contrast with the results of Gao et al,[9] whose work suggests that CNVs are an early event during tumor evolution. Deletion of key genes are important for tumor progression and metastases. In our work, *TET1*, a gene that encodes a cytosine demethylase, was the most frequently deleted in mTNBC (8.6%). *TET1* expression decreases cell invasion and tumor formation in human breast cancer cell line xenografts, inhibiting DNA methylation of tissue inhibitors of the metalloproteinase family proteins.[22]

The hallmarks of cancer describe 10 biological capabilities from tumors acquired during their evolution.[23] In mTNBC, genes whose alterations were more recurrent are related to the hallmark "genomic instability and mutation," this is explained by the high incidence of *TP53* and *BRCA1* mutations in pTNBC.[3] On the contrary, the hallmark "avoiding immune destruction" was the least represented among gene alterations in mTNBC (Figure 1). Our results suggest that the activation

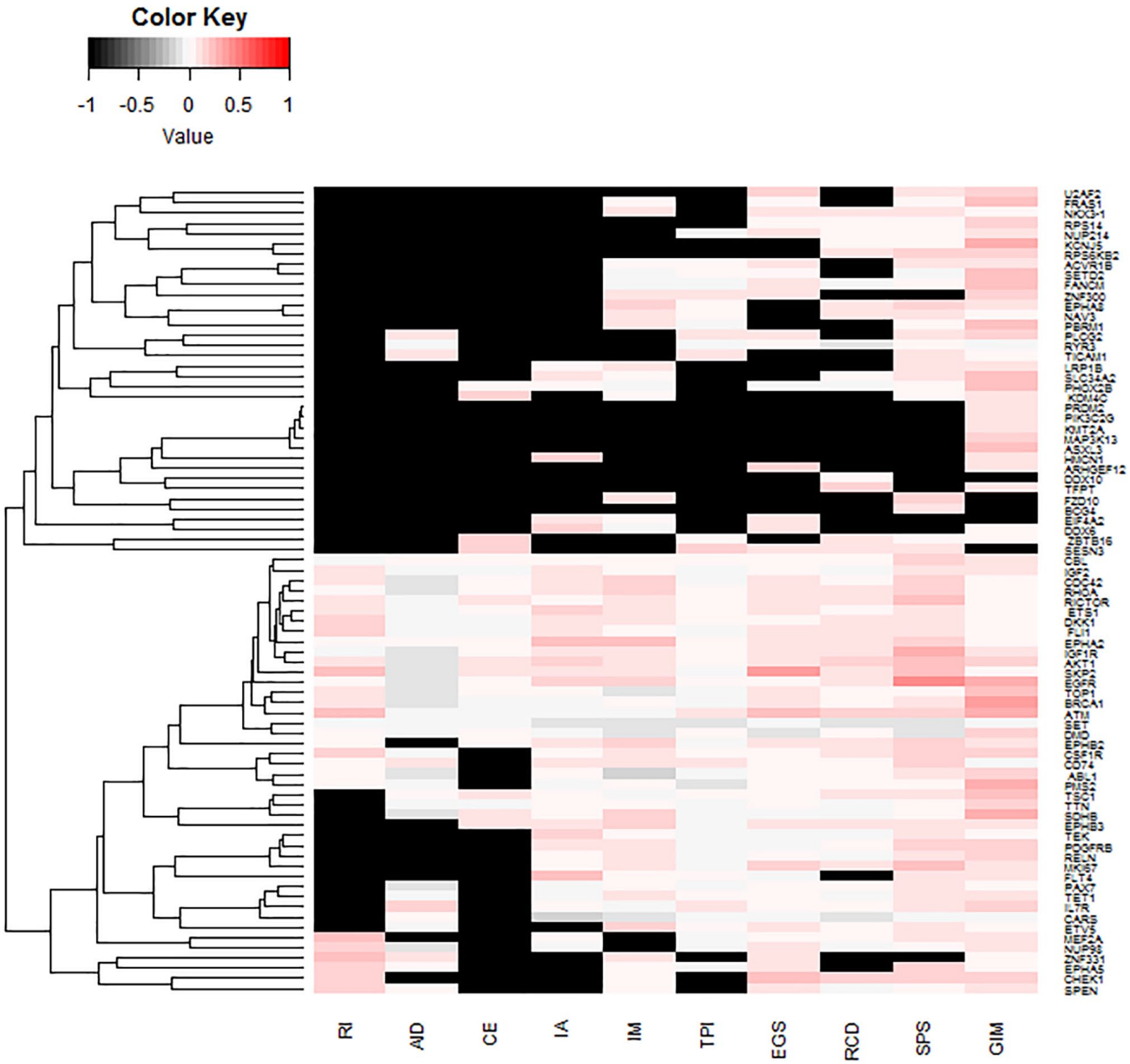

Figure 1. Hallmarks of cancer enriched in mTNBC.
Values of normalized pointwise mutual information (NPMI) were estimated using the software Cancer Hallmarks Analytical Tool.
Abbreviations: AID, avoiding immune destruction; EGS, evading growth suppressors; GIM, genome instability and mutation; IA, inducing angiogenesis; IM, Invasion and metastasis; RCD, resisting cell death; RI, replicative immortality; SPS; sustaining proliferative signaling; TPI, tumor promoting inflammation.

of this hallmark is an early event that does not need to be significantly increased during metastases.

Finally, our data of protein-protein interaction indicates that at least three gene clusters with alterations are prevalent in mTNBC (Figure 2). These clusters involve genes related to DNA repair, in concordance with the overrepresentation of the hallmark of "genomic instability and mutation." In addition, evidence of increased metabolism is observed through the enrichment of alterations in genes participating in the mTOR signaling pathway. The third cluster of genes involves well-known tyrosine kinase receptors such as *EGFR, PDGFR*, and others.

The presence of genes encoding Eph receptors in the interactions is interesting. In our analysis, we observed that the loss of this family of receptors is common in mTNBC. Although typically, Eph receptors promote cell division and are commonly overexpressed in human tumors and have a dual role in tumor promotion and suppression.[24,25]

In conclusion, despite the limitations of this study, we could identify genes and potential mechanisms involved in TNBC evolution through metastasis. Our analysis identified that deletions were the most frequently enriched alterations and also suggests that some biological mechanisms are potentially targetable.

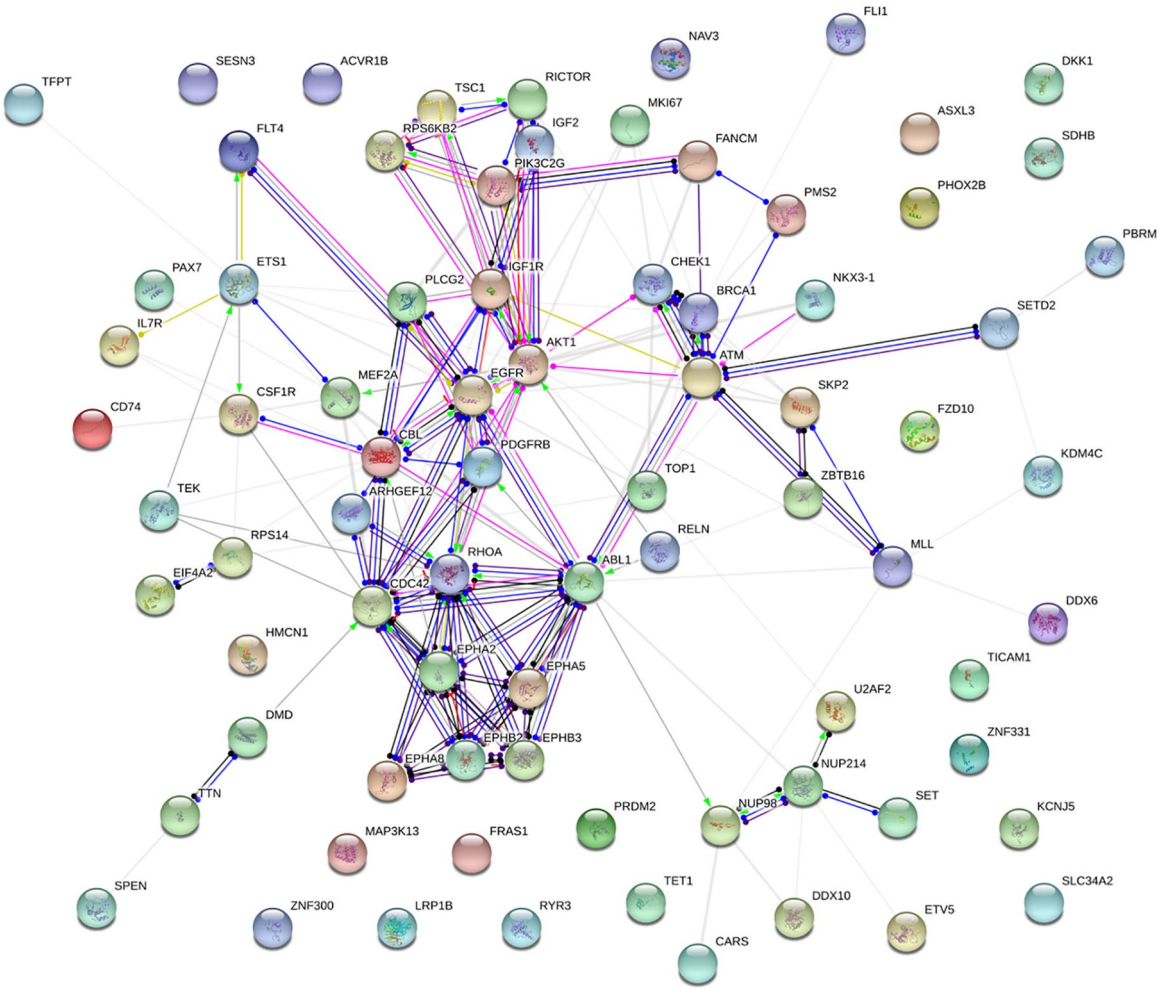

Figure 2. Prediction of protein-protein interaction among gene alterations significantly enriched in mTNBC.

Author Contributions

Study design: C.H.S, C.F, L.J.S and J.A.P. Data collection, data assembly and data preprocessing: C.H.S, L.B, J.Z, J.A. Statistical Analysis: C.F. and S.N. Data interpretation: all authors. Writing of Manuscript: All authors. Preparation of tables and figures: C.H., L.B, J.Z and J.A. All authors reviewed and approved the manuscript.

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