Copy Number Variation in Triple Negative Breast Cancer Samples Associated with Lymph Node Metastasis

Mamta Pariyar
  Newcastle University Faculty of Medical Sciences

Andrea Johns
  University of Newcastle

Rick Francis Thorne
  University of Newcastle

Rodney J. Scott
  University of Newcastle

Kelly A. Avery-Kiejda (✉ kelly.kiejda@newcastle.edu.au)
  University of Newcastle

Research article

Abstract

Background

Triple negative breast cancer (TNBC) is a highly metastatic and aggressive subtype of breast cancer and cases presenting with lymph node involvement have worse outcomes. This study aimed to determine the regions of copy number variation (CNV) associated with lymph node metastasis in TNBC patients.

Methods

CNV analyses were performed in a study cohort of 23 invasive ductal carcinomas (IDC), 12 lymph node metastases (LNmets) and 7 normal adjacent tissues (NAT); as well as in a second cohort containing 70 TNBC IDC samples and the same 7 NATs. CNVs associated genes were analysed using GO-enrichment and Pathway analysis and also integrated with gene expression analysis. The prognostic role for genes showing CNV based change in mRNA expression was determined using Kaplan-Meier (KM) plotter database.

Results

For the IDCs, frequent amplification was evident in chromosomal regions 8q, 1q, 2(p and q), 3q24, 10p and 12p and deletion in 3p, 4 (p and q), 5q, 14q and 17p in contrast to LNmets showing frequent amplification of 4q, 2p, 3q24, 1q, 10p, 12p, 8q, 20p, 21q and 6p and deletion in 1p, 4q (4q21.1, 4q26) and 5q. A total of 686 (441 amplified and 245 deleted) genes were associated with lymph node metastasis. The LNmet-associated genes were highly enriched for "regulation of complement activation", "regulation of protein activation cascade", "regulation of humoral immune response", "oxytocin signalling pathway" and "trail binding" pathways. Moreover, 6/686 LNmet-associated genes showed CNV-based changes in their mRNA expression of which, high expression of ASPM and KIF14 was significantly associated with worse relapse free survival.

Conclusion

This study has identified several CNV regions in TNBC that could play a major role in metastasis to the lymph node.

1. Background

Breast cancer is the second most common cancer among women worldwide as well as a major cause of cancer-related deaths (1). Triple negative breast cancer (TNBC) is characterized by a lack of two hormonal receptors: progesterone receptor (PR) and estrogen receptor (ER), as well as a lack of overexpression of human epidermal growth factor receptor-2 (HER2) (2). It comprises about 15−20% of all the breast cancers cases (3); and is one of the most aggressive breast cancer subtypes, as it metastasizes rapidly and often recurs within 3−5 years after diagnosis when compared to ER-positive breast cancer, which typically recurs at later stages (beyond 5 years) (4−6). Furthermore, if a women

survives with disease progression for approximately five years her risk of recurrent disease is similar to that of women diagnosed with ER + PR + ve disease (4). There is no biomarker that can discriminate between women who will do well, those who develop recurrent disease nor are there targeted therapies for this breast cancer subtype, and therefore, cytotoxic chemotherapy and surgery are the only options for the treatment of TNBC. Although, PARP inhibitors and EGFR inhibitors are in clinical trials, consistent results have not been obtained (7). Identification of target molecules is essential to enable better prognostic indicators for this disease or new targets for therapy in order to improve patient survival.

Copy number variation (CNV) is a type of structural variation where the DNA sequence, ranging from 1 kb to several megabases in length, is either amplified or deleted compared to the normal copy. It is one of the major sources of human genetic variation (8), and it is associated with the initiation of cancer and other diseases such as cardiovascular and complex neurological illnesses (9–11). CNVs contribute 4.8–9.5% of the variability in the human genome (12). One research finding suggests that 62% of highly amplified genes in breast cancer exhibit at least a 2-fold increase in expression (13).

Several studies have shown that TNBCs with lymph node involvement have a higher probability of recurrence and worse survival (14–16). Additionally, in TNBC, some CNVs can predict poor outcomes and act as prognostic factors (17–19). TNBC has greater genomic instability than other non-TNBC subtypes (20) and it is likely that some recurrent CNVs in TNBC may be related to lymph node metastases. There are few studies of CNV based genes associated with lymph node metastasis in breast cancers. The frequent amplification of myeloid cell leukemia sequence 1 (MCL1) was found in primary tumours with lymph node metastasis but absent in primary tumours without metastasis in ER positive breast cancer as determined by single cell sequencing (21). Gains in CTAGE5 were associated with lymph node metastases in breast cancer using TCGA and METABRIC data from breast ductal carcinoma (22), whereas, in TNBC, there have been no previous studies of CNVs that are associated with lymph node metastasis.

Previous studies from our laboratory were performed to explore gene, miRNA and methylation changes, aimed at identifying potential biomarkers of progression in TNBC (23–25). Differentially expressed genes in tumour and lymph node metastasis compared to normal adjacent tissues were identified and 39% of the genes were associated with altered methylation levels, whereas a large proportion of the differentially expressed genes (61%) were not associated with altered methylation levels in invasive ductal carcinomas (IDC) vs normal adjacent tissues (NAT) (23). Since CNVs are a frequent event in cancer, the differential expression of these genes in TNBC may be due to a variation in copy number.

In this study, we performed CNV analysis to define regions of copy number gain or loss in TNBC. Genes overlapping the regions of CNV were compared with their mRNA expression levels. Moreover, by comparing CNV-based genes in LN positive TNBCs and LN metastases, common genes were identified which were associated with the progression of this disease to LN metastasis.

2. Methods

2.1. Study and second cohorts

In total 23 invasive ductal carcinomas (IDCs), 12 lymph node metastases (LNmets), 3 pooled NAT samples and 1 singular NAT (11samples in total) from TNBC cases previously used for methylation analysis were used for CNV analysis. One pooled NAT sample was removed in this CNV analysis after quality control analysis (leaving 7 NAT samples in total for the analysis). As previously described (23, 24), these samples were obtained from formalin fixed paraffin-embedded tissues from the archives of NSW Health Pathology North, John Hunter Hospital, Newcastle, Australia. Areas of IDC, LNmets and NAT were identified and confirmed by pathologist. A second cohort was used for copy number analysis and contained 70 TNBC samples from the Australian Breast Cancer Tissue Bank (ABCTB), which have been previously described (23) and these were compared to the same 7 NAT samples used in the study cohort.

2.2. DNA extraction

As previously described, the Gentra Puregene Tissue Kit (Qiagen,Venlo,Limburg, Netherlands) was used to extract DNA from FFPE tissues (23).

2.3. Illumina Infinium HD FFPE methylation arrays

Methylation array analysis was performed using Human Methylation 450K BeadChip arrays (Illumina) as previously described (23). The results were deposited in Gene Expression Omnibus (Accession No. GSE78751).

2.4. Gene expression microarray analysis

Human Gene 2.0 arrays were used for gene expression analysis and data analysis was performed using Genomic Suite 6.6 (Partek) as previously described (25). The gene expression results were deposited in Gene Expression Omnibus (Accession No. GSE61723).

2.5. CNV analysis

Previous studies from our laboratory performed Illumina Human Methylation 450K BeadChip arrays on the same cohorts as used in this study (23). These arrays can also be used to detect CNVs as they are a dense SNP array (26). Therefore, in the current study, we performed CNV analysis using the signal intensity data generated from the previous methylation data.

In total 23 IDCs, 12 LNmets and 7 NAT samples in the study cohort were used for copy number analysis. Similarly, in the second cohort, 70 IDCs and 7 NAT samples were used for copy number analysis. In

addition, of the 23 IDCs described in the primary cohort, 13 are LN negative and 10 are LN positive samples, these were used to compare against the 12 LNmets to determine CNVs associated with LNmet.

The idat files were imported into Partek Genomic Suite 7.0 Software. The probe intensity data was parsed from the idat files. Copy number was created using 7 NAT samples as a reference baseline and values were converted to log2 ratios. Following that, the values were adjusted for local GC content at a window size of 1 Mb to reduce genomic waviness (27) .

A genomic segmentation algorithm was used for copy number detection to identify copy number changes between two neighbouring regions. This algorithm was used with the settings of minimum genomic markers >= 40, signal to noise ratio = 0.3 for the magnitude of significant region differences relative to the noise level in each sample and a P-value threshold = 0.001 for significance between different regions. A segment was considered as amplified if the mean copy number was >=0.2 and a deletion if the mean copy number was <=-0.3. A false discovery rate (FDR) of 0.05 was applied to the P-value to account for multiple testing.

The CNV regions with amplification and deletion across the genome shared by at least five samples were selected for further analysis in IDCs of the study cohort and the same proportion of cases for selection was used for IDCs in the second cohort (28, 29). Ensembl Transcript release 75 database was used to determine the genes located in those CNV regions.

GO-enrichment and pathway analysis within Partek Genomic Suite was used to identify enriched GO terms and pathways from the list of copy number altered genes. Enrichment scores > 3 were considered significant and a q-value of <0.05 was applied to the P-value for multiple test correction. The list of copy number altered genes in IDCs and LNmets of the study cohort were compared with gene expression data previously performed on the study cohort to determine if there were CNV based changes in expression of the genes (25).

2.6. Kaplan Meier survival analysis

The KM plotter database was used for survival analysis which contains 255 TNBC samples (30). The database possesses relapse free survival and overall survival information downloaded from Gene Expression Omnibus (GEO), European genome-phenome Archive (EGA) and The Cancer Genome Atlas (TCGA). High and low expression groups of genes of interest were divided according to "Auto select best cut-off" which is the cut-off value of most statistically significant from all possible cut-off values computed between lower and upper quartiles of the expression. Relapse free survival analysis was performed and a hazard ratio with 95% confidence interval and Log-rank P-values were calculated within the database.

3. Results

3.1. CNVs in IDCs and LNmets compared to NAT samples

CNV analysis was performed on the study cohort of 23 IDCs, 12 LNmets and 7 NAT samples. This analysis identified 4709 CNV segments in IDCs when compared to NAT, with 65% associated with gains and 35% with losses (Table S1). Similarly, in LNmets, a total of 1725 CNV segments were identified with 58.9% associated with gains and 41.1% losses (Table S2), which were not present in NATs. All CNV segments ranged from more than one kilobase to several megabases in size.

Similarly, in the second cohort of 70 TNBC IDCs and the 7 NAT samples, many more regions were amplified rather than deleted. In total, 15597 CNV segments were identified in IDCs with 61.59% gains and 38.4% losses, which were not present in NATs (Table S3).

3.2. Gene annotation of CNVs in IDCs of the study and second cohorts

In IDCs of the study cohort, the most recurrent amplified regions were in the large areas of 8q (such as 8q24.21, 8q23.3, 8q24.13, 8q23.2-8q23.3, 8q23.3, 8q23.3-8q24.11, 8q24.11, 8q24.11-8q24.12, 8q24.13, 8q24.13-8q24.21, 8q24.22-8q24.23, 8q24.3 and 8q22.3) (Table S4.a), followed by 1q (such as 1q21.2, 1q21.3, 1q21.2- 1q21.3, 1q21.3, 1q23.3, 1q24.2), 10p (10p15.3, 10p15.2-10p15.1), 12p13.1-12p12.3 and 2q33.1. After these regions were overlapped with gene annotation data, 594 genes were identified in 8q, which were frequently amplified in more than 50% of the 23 samples (Table S4.b). In Figure 1A, the histogram height showed that the majority of samples shared amplification (red) across the 8q region. Whereas, only 78 genes in 1q, 7 in 2q33.1, 29 in 10p15.1-10p15.3 and 10 in 12p13.1-12p12.3 were frequently amplified in more than 50% of the 23 samples. The most recurrent deleted regions were 14q24.1, followed by 14q21.1, 19p13.3, 4q34.1, 5q13.2 and 5q32 (Table S5.a). Figure 1A shows the higher distribution of deletion (blue) across the q regions of chromosome 4, 5, 14 and 19p compared to the other chromosomal regions. However, deleted regions were shared less among the samples compared to that of the amplified regions. Four genes in 14q24.1, two in 14q21.1, two in 19p13.3, two in 4q34.1, two in 5q13.2 and five in 5q32 were highly recurrent, observed in more than ~40% of the samples (Table S5.b).

To investigate if genes overlapping the amplified and deleted regions in IDCs of the study cohort were associated with specific functional groups and pathways, GO-enrichment and Pathway analysis was used. According to the enrichment scores, "production of molecular mediator of immune response", "antigen binding", "immunoglobulin production", "complement activation" and "regulation of protein activation cascade" were the most enriched GO terms (Table S6.a) and "ribosome biogenesis in eukaryotes" was the most enriched pathway from the list of genes contained within the amplified regions (Table S6.b). In concordance with this finding, upregulation of "gene networks related to ribosome biogenesis" has previously been demonstrated in the MDA-MB-231 TNBC cell line (31).The list of genes overlapping the deleted regions, were highly enriched in GO terms such as "neuron differentiation",

"Cardiac ventricle morphogenesis", "cardiac chamber morphogenesis", "system development" and "regulation of hormone levels" (Table S6.c). No pathways were significantly enriched in the list of deleted genes.

In the second cohort, the most frequent amplified regions were 3q24, 2p15, 6p22.1, 8q (8q24.3, 8q11.1, 8q23.2-8q23.3), 1q (1q44, 1q42.2), 10p15.3 and 4q28.3 (Figure 2) (Table S7.a). While at the gene level, 47 genes in 8q, 43 in 1q, 15 in 10p15.3, 5 in 4q28.3, 3 in 3q24, 1 in 2p15, 1 in 6p22.1 were amplified in more than 50% of samples. The most frequent deleted regions were 17p13.1 and 3p21.31. Only 8 genes in 17p13.1 and 7 in 3p21.31 were observed to be frequently deleted in more than 39% of the samples (Table S7.b).

Similar to the study cohort, in IDCs of the second cohort, the most significantly overrepresented GO terms in the list of genes which were amplified were "antigen binding", "immunoglobulin production", "production of molecular mediator of immune response", "complement activation", whereas "ribosome biogenesis in eukaryotes" was a significantly enriched pathway (Table S8.a and S8.b). In the list of deleted genes, "neutrophil mediated cytotoxicity", "neutrophil mediated killing of symbiont cell", "neutrophil mediated immunity" and "killing by host of symbiont cells" were the most enriched GO terms (Table S8.c).

Interestingly, 2601 of total 8943 (29%) CNV associated genes in study cohort were detected in the second cohort showing good concordance between the cohorts. Of the 2601 genes, 2535 were amplified and 66 were deleted in both cohorts. 1599 of 2535 (63%) amplified genes were associated with the chromosome 1q, followed by 303 in 8q, 282 in 19(p and q), 114 in 2p, 68 in 5p while rest the fewer genes were distributed across the 3q, 4q, 6p, 7(p and q), 10p, 12p, 17q, 18p and 20q (Table S9.a). Of the 66 deleted genes in both cohorts, 37 were associated with 8p followed by 22 in 5q, 3 in 19p13.3 while the rest were associated with 3p21.31, 4q32.3, 14q13.2 and 17p13.1 (Table S9.b).

The most enriched Go terms were "immunoglobulin production" and "antigen binding" and "ribosome biogenesis in eukaryotes" was the enriched pathway in the list of amplified genes (Table S10.a and S10.b). While in the list of deleted genes, neutrophil mediated cytotoxicity, "cellular extravasation" and "regulation of chemokine biosynthetic process" were the most enriched Go terms (Table S10.c).

3.3. Gene annotations of CNVs in LNmets

The most frequently amplified regions in the LNmets were 4q28.3, 2p (2p15, 2p11.2-2p11.1), 3q24, 1q21.2, 10p (10p15.3, 10p15.2), 12p11.1, 8q (8q11.1, 8q21.13-8q21.2, 8q24.21, 8q23.3), 20p11.22-20p11.21, 21q22.13, 6p22.1 (Table S11.a, Figure 1B). At the gene level, 2p contained the highest number of amplified genes (105 genes) (Table S11.b) and accounted for more than 50% of cases; followed by 19 in 8q and 15 in 20p11.22 - 20p11.21; whereas 3 in 1q21.2, 6 in 10p15.3, 5 in 12p11.1, 2 in 21q22.13, 4 in 3q24, 6 in 4q28.3, 3 in 6p22.1 were frequently observed in more than 50% of samples. The most frequently deleted regions were 1p36.23, 4q21.1, 5q (5q11.2, 5q23.2) in more than 39% of the samples.

Similar to IDCs, the number of deleted regions shared among the multiple samples was less than that of the amplified regions. There were 28 genes in 5q, 7 in 4q21.1 and 5 in 1p36.23 deleted in more than 39% of samples (Table S11.c).

The most enriched GO terms were "Production of molecular mediator of immune response", "Immunoglobulin production", "antigen binding", "regulation of protein activation cascade" and "regulation of complement activation" and "ribosome biogenesis in eukaryotes" was the most enriched pathway in the list of amplified genes (Table S12.a, S12.b). The list of deleted genes showed highest enrichment in GO terms such as "flavonoid glucuronidation", "flavonoid metabolic process", "cellular glucuronidation" and "glucuronate metabolic process". Other highly enriched pathways in the list of deleted genes were mainly involved in metabolism such as "Pentose and glucuronate interconversions", "steroid hormone biosynthesis", "drug metabolism", "chemical carcinogenesis" and the "estrogen signaling pathway" (Table S12.c, S12.d).

3.4. Genes within CNV regions associated with the progression from IDC to LN metastasis

We next determined the genes associated with CNVs in the LNmets to identify changes related to the progression of primary TNBC to metastasis in the study cohort. For this, first we identified total CNV regions of each group and the genes associated to that region. Then we compared the amplified and deleted genes amongst the three groups using Venn diagrams. Group 1: Lymph node positive IDC (IDC LN+) (n=10), Group 2: Lymph node negative IDC (IDC LN-) (n=13) and Group 3: Lymph node metastases (LNmets) (n=12). With this comparison, we aimed to identify genes in common with copy number alterations in IDC LN+ and LNmets, that were not present in IDC LN-, that were potentially associated with metastasis (Figure 3A and 3B).

We identified 441 amplified genes located in chromosome 1q, 5p, 6(p and q), 7q, 8(p and q), 17q and 20q that were in common with IDC LN+ and LNmets. Interestingly, 365 of 441 (83%) genes were associated with the q region of chromosome 1, whilst 30 of 441 (7%) genes were associated with chromosome 6 (6p22.1, 6p24.3 - 6p24.2 and 6q21) and 26 of 441(6%) genes in the 17q region (17q23.3 and 17q25.3) (Table 13.a). Two hundred and forty five deleted genes were located on chromosome 5q, 6p, 8p, 12q, 14q, 17q and 19p and were common to both IDC LN+ and LNmets. Here, 146 of 245 (60%) deleted genes were located in 8p, followed by 50 in 5q (20.4%), 32 in 14q (13.06%), with the other regions encompassing less than 10 genes. The CNV-altered genes that are present in both IDCs LN+ and LNmets but not in IDC LN- are potentially involved in metastatic TNBC disease (Table S13.b).

Performing GO-enrichment and Pathway analysis, the list of amplified genes showed highest enrichment in GO terms in pathways associated with "regulation of complement activation", "protein activation cascade", "regulation of acute inflammatory response", "regulation of protein processing and maturation" and "humoral immune response"; and the highest enrichment in pathways including "complement and

coagulation cascades" and "oxytocin-signalling pathway" (Table S14.a, S14.b). Whereas, "TRAIL binding" was the most enriched GO term while "estrogen signalling pathway" and "cytokine-cytokine receptor interaction" were significantly associated with the deleted regions. (Table S14.c, S14.d).

3.5. Integration of CNVs with gene expression analyses

CNV data was integrated with previously published gene expression data (GEO Accession: GSE61723) to determine whether the change in mRNA expression was a result of the CNVs (25). However, very few differentially expressed genes were linked to the CNVs in the study cohort.

In the IDCs of study cohort, we identified 33 of 185 (18%) differentially expressed genes in IDC vs NAT that were copy number altered, where 29 significantly upregulated genes in IDC vs NAT were amplified and 4 significantly downregulated genes in IDC vs NAT were deleted (Figure 4A and 4B) (Table S15).

In the LNmets, 18 of 165 (10.9%) differentially expressed genes in LNmet vs NAT showed copy number alterations, where 5 upregulated genes were amplified and 13 downregulated genes were deleted (Figure 4C and 4D) (Table S16).

Our previous study identified 28 TNBC specific genes that were differentially expressed in IDCs vs NAT of study cohort but not in non-TNBC IDCs (25). In the current study, 3 of 28 TNBC specific genes whose expression was upregulated, were amplified in IDCs of the study cohort (ANKRD36BP1, ANP32E, MYBL1), whereas TBC1D9 and TMEM144 whose expression was downregulated, were deleted in the IDCs of the study cohort.

Additionally, we investigated if the 441 genes which were amplified in both IDC LN+ and LNmets and 245 genes which were deleted in both IDC LN+ and LNmets in our current study also showed differential expression in IDC LN+ vs NAT. For this, we compared these genes with the total 104 genes that were differentially expressed in IDC LN+ vs NAT. The total 104 genes is the result from the previous study (25) which was not published. Only three amplified (ASPM, KIF14, LEMD1) genes were upregulated and three (SNORD113-2, SNORD113-3, SNORD113-4) deleted genes were downregulated in IDC LN+ vs NAT (Table 1).

3.6. CNVs associated with prognosis

We evaluated the prognostic value of the LNmets associated genes located in regions of CNV, which showed a corresponding change in mRNA expression in the study cohort. Relapse free survival analysis (RFS) was performed for three of 441 amplified genes and for three of 245 deleted genes which were associated with LNmet and showed a corresponding change in mRNA expression. Of the three amplified genes, high expression of ASPM and KIF14 were significantly associated with worse RFS (Figure 5), while high expression of LEMD1 showed a non-significant trend in increased RFS. No survival information was available for the three (SNORD113-2, SNORD113-3, SNORD113-4) deleted genes.

4. Discussion

TNBC is an aggressive subtype of breast cancer that has an increased rate of metastasis and poor clinical outcomes when compared to other subtypes. It is very heterogeneous and associated with a higher burden of CNVs compared to other subtypes (32). There are no studies that have performed analysis of CNVs that are associated with LNmet in TNBC, we aimed to identify CNVs which may play a role in metastases of this subtype. We have identified the most frequent CNVs in IDCs and LNmets and the overlapping genes. We have also identified CNV-based genes which are LNmet-associated by comparing the groups of LN positive IDCs, LN negative IDCs and LNmets.

The most frequent amplification in chromosomal regions was 8q, 1q, 2 (p and q), 3q24, 10p and 12p and deletions in 3p, 4 (p and q), 5q, 14q and 17p of the IDCs. The most frequent amplification in LNmets was in chromosomal regions 4q, 2p, 3q24, 1q, 10p, 12p, 8q, 20p, 21q and 6p, whereas deletions were identified in 1p, 4q (4q21.1, 4q26) and 5q. Similar to our results, other studies have also found frequent amplification in 1q, 3q, 8q, 10p and 12p and deletion in 5q and 17p in TNBC (33, 34) suggesting these CNV regions may have a significant role in increasing genomic aberrations in TNBC.

We have identified a total 686 (441 amplified and 245 deleted) CNV-based genes which were observed in both LN positive IDCs and LNmets and that were not present in LN- IDCs; and thus, are associated with metastasis to the lymph node. This implies that certain CNVs are shared between the primary tumour and the metastasis, which could be involved in driving metastatic progression. Interestingly, we observed that the majority of the LNmet-associated genes were located in 1q amplified and 8p deleted regions in our study. The gain in 1q has also been associated with metastasis in breast cancer (21, 35) as well as in multiple other cancers such as renal clear cell carcinoma and papillary thyroid carcinoma (36, 37). Amplified genes in the chromosome 1q region identified included CD55, CR1, CR2, CD46 and C4BPB, also known as complementary regulatory proteins that are known to be overexpressed in cancer cells and promote lymph node metastasis in various cancer types such as nasopharyngeal, gastric and pancreatic (38–41). In our study, we have identified the highest number of LNmet-associated deleted genes located in 8p region. The deletion of 8p was also associated with metastasis in hepatocellular carcinoma (42). One study reported the loss of 8p in the invasive micropapillary carcinoma cases (IMC) detected by comparative genomic hybridization (43). Moreover, loss of 8p was significantly linked to the presence of lymph node metastasis in breast cancer (44). These suggest that the gain in 1q and deletion of 8p may have a significant role in metastasis.

Furthermore, regulation of 'complement activation cascade' and 'humoral immune response' were highly enriched in the LNmet-associated genes that were amplified indicating the genomic imbalance in complement activation and humoral immune response may assist the tumour to escape immune attack leading to its progression and metastasis. CD55 and CD46 which are amplified in our study, are found to regulate the immune response (45, 46) but the alteration of these proteins may dysregulate the immune mechanism which could enhance metastasis (47). We observed 'TRAIL binding' was highly enriched within the list of deleted genes. Upon binding to TRAIL Receptor 2 (TRAIL-R2), TNF-related apoptosis-

inducing ligand (TRAIL) and agnostic mAbs have been shown to act as a metastasis suppressor in an orthotropic model of TNBC (48). Deletion of TNFRSF10C (TRAIL-R3), also identified in our study, was associated with distant metastasis and positive nodal disease in colorectal cancer (49). Overall, our results suggest the dysregulation of the immune response and the apoptotic pathway may play a significant role in regulating metastasis of the primary tumour.

Moreover, we found 6 of the total 686 (441 amplified and 245 deleted) LNmet-associated genes present in both IDC LN + and LNmets also showed CNV based changes in their expression. Of the 6 LNmet associated genes, SNORD113-2, SNORD113-3, SNORD113-4 were both deleted and downregulated in IDC LN + vs NAT. These three genes also known as small nucleolar RNAs (snoRNAs) belong to a group of non-coding RNA (ncRNA) molecules which play a role in the ribosomal RNA biogenesis (50, 51). However, the role of SNORD113-2, SNORD113-3, SNORD113-4 has not been studied in TNBC. One study has shown that the downregulation of SNORD113-1, which belongs to the same family of snoRNAs, is associated with worse relapse free survival in hepatocellular carcinoma (52). The remaining three LNmets associated genes (ASPM, KIF14 and LEMD1) were both amplified and upregulated in IDC LN + vs NAT. ASPM is known for its role in spindle microtubule organization in cell division (53). The high expression of ASPM was also observed in several other cancers such as bladder cancer (54), hepatocellular carcinoma (55), gliomas (56), ovarian cancer (57) and prostrate cancer (58) where the increase in expression was mostly associated with tumour grade, early recurrence, tumour metastasis and worse survival. Moreover, knockdown of ASPM in pancreatic cancer cell lines reduced cell proliferation and migration (59). From KM plotter survival analysis, we found the high expression of ASPM is associated with worse relapse free survival (RFS) in TNBC (30). KIF14 is responsible for mitotic spindle formation and cytokinesis and has been associated with decreased disease-free survival in breast and lung cancer (60, 61). High expression of KIF14 has also been found in other cancers such as prostate cancer (62), ovarian cancer (63) and hepatocellular carcinoma (64), where it is associated with tumour growth and worse survival. Furthermore, we found the high expression of KIF14 in our study is associated with worse relapse free survival (RFS) in TNBC, in agreement with these studies (30). LEMD1 overexpression was associated with nodal metastasis and worse prognosis in oral squamous cell carcinoma (65) and in gastric cancer (66). Overall, these genes with CNV-associated changes in expression have a significant role in multiple cancers and may also play a greater role in increasing disease aggressiveness in TNBC. Although for many of the 686 genes there was no association between CNV and gene expression, this may be due to the various factors including the degree of overlap of CNVs in genes, distance to transcription start sites and types of genes. One study showed that the genes associated with the regions amplified discontinuously are downregulated suggesting partial gene amplification may acts as the silencer to downregulate the genes expression (67).

Although this study is limited by the small sample size of LNmets, a series of CNVs has been identified that appear to be associated with LNmet which contains genes that have a central function in maintaining a number of key pathways, which, if perturbed, result in an increased departure from the normal mechanisms that are associated with mammary homeostasis.

5. Conclusions

This study has identified several regions of CNV in TNBC that could play major role in metastasis to the lymph node. Further validation of these CNVs in a larger cohort and functional studies are necessary to understand their role in the progression of TNBC.

6. List Of Abbreviations

TNBC
Triple negative breast cancer
CNV
Copy number Variation
FFPE
Formalin Fixed embedded tissues
IDC
Invasive ductal carcinomas (IDC),
LNmets
lymph node metastases
NAT
normal adjacent tissues

7. Declarations

7.1. Ethics approval and consent to participate

This study complies with the Helsinki Declaration with ethical approval from the Hunter New England Human Research Ethics Committee (Approval number: 09/05/20/5.02). In accordance with the National Statement on Ethical Conduct in Research Involving Humans, a waiver of consent was granted for the study cohort. Written informed consent was obtained from all patients in the second cohort (23).

7.2. Consent for publication

Not Applicable

7.3. Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

7.4. Conflicts of Interest

The authors declare no conflict of interest.

7.5. Funding

7.6. Author Contributions

Conceptualization, K.A.A.K and M.P; data curation, M.P, A.M, K.A.A.K; methodology; M.P, A.M, K.A.A.K; writing—original draft preparation, M.P.; writing—review and editing, M.P, A.M, R.F.T, R.J.S, K.A.A.K; supervision, R.F.T, R.J.S, K.A.A.K.; funding acquisition, R.J.S., K.A.A.K. All authors have read and agreed to the published version of the manuscript

8. References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.

2. Anders C, Carey LA. Understanding and treating triple-negative breast cancer. Oncology (Williston Park). 2008;22(11):1233–9. discussion 9–40, 43.

3. Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. Clin Breast Cancer. 2009;9(Suppl 2):73–81.

4. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13(15 Pt 1):4429–34.

5. Kennecke HFOI, Speers C, et al. Late risk of relapse and mortality among postmenopausal women with estrogen responsive early breast cancer after 5 years of tamoxifen. Ann Oncol. 2007;18(1):45–51.

6. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol. 2008;26(8):1275–81.

7. Schmadeka R, Harmon BE, Singh M. Triple-negative breast carcinoma: current and emerging concepts. Am J Clin Pathol. 2014;141(4):462–77.

8. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, et al. Global variation in copy number in the human genome. Nature. 2006;444(7118):444–54.

9. Walker LC, Pearson JF, Wiggins GA, Giles GG, Hopper JL, Southey MC. Increased genomic burden of germline copy number variants is associated with early onset breast cancer: Australian breast cancer family registry. Breast cancer research: BCR. 2017;19(1):30.

10. Vijay A, Garg I, Ashraf MZ. Perspective: DNA Copy Number Variations in Cardiovascular Diseases. Epigenet Insights. 2018;11:2516865718818839.

11. Zhang F, Gu W, Hurles ME, Lupski JR. Copy number variation in human health, disease, and evolution. Annu Rev Genomics Hum Genet. 2009;10:451–81.

12. Zarrei M, MacDonald JR, Merico D, Scherer SW. A copy number variation map of the human genome. Nat Rev Genet. 2015;16(3):172–83.

13. Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, et al. Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. Proc Natl Acad Sci U S A. 2002;99(20):12963–8.

14. Mousavi SA, Kasaeian A, Pourkasmaee M, Ghavamzadeh A, Alimoghaddam K, Vaezi M, et al. Assessing the prognostic factors, survival, and recurrence incidence of triple negative breast cancer patients, a single center study in Iran. PLoS One. 2019;14(1):e0208701.

15. Gonçalves H Jr, Guerra MR, Duarte Cintra JR, Fayer VA, Brum IV, Bustamante Teixeira MT. Survival Study of Triple-Negative and Non-Triple-Negative Breast Cancer in a Brazilian Cohort. Clinical Medicine Insights Oncology. 2018;12:1179554918790563-.

16. Ovcaricek T, Frkovic SG, Matos E, Mozina B, Borstnar S. Triple negative breast cancer - prognostic factors and survival. Radiol Oncol. 2011;45(1):46–52.

17. Kikuchi-Koike R, Nagasaka K, Tsuda H, Ishii Y, Sakamoto M, Kikuchi Y, et al. Array comparative genomic hybridization analysis discloses chromosome copy number alterations as indicators of patient outcome in lymph node-negative breast cancer. BMC Cancer. 2019;19(1):521.

18. Nedeljkovic M, Tanic N, Dramicanin T, Milovanovic Z, Susnjar S, Milinkovic V, et al. Importance of Copy Number Alterations of FGFR1 and C-MYC Genes in Triple Negative Breast Cancer. J Med Biochem. 2019;38(1):63–70.

19. Park HS, Jang MH, Kim EJ, Kim HJ, Lee HJ, Kim YJ, et al. High EGFR gene copy number predicts poor outcome in triple-negative breast cancer. Mod Pathol. 2014;27(9):1212–22.

20. Shah SP, Roth A, Goya R, Oloumi A, Ha G, Zhao Y, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature. 2012;486(7403):395–9.

21. Bao L, Qian Z, Lyng MB, Wang L, Yu Y, Wang T, et al. Coexisting genomic aberrations associated with lymph node metastasis in breast cancer. J Clin Investig. 2018;128(6):2310–24.

22. Behring M, Shrestha S, Manne U, Cui X, Gonzalez-Reymundez A, Grueneberg A, et al. Integrated landscape of copy number variation and RNA expression associated with nodal metastasis in invasive ductal breast carcinoma. Oncotarget. 2018;9(96):36836–48.

23. Mathe A, Wong-Brown M, Locke WJ, Stirzaker C, Braye SG, Forbes JF, et al. DNA methylation profile of triple negative breast cancer-specific genes comparing lymph node positive patients to lymph node negative patients. Sci Rep. 2016;6:33435.

24. Avery-Kiejda KA, Braye SG, Mathe A, Forbes JF, Scott RJ. Decreased expression of key tumour suppressor microRNAs is associated with lymph node metastases in triple negative breast cancer. BMC Cancer. 2014;14:51-.

25. Mathe A, Wong-Brown M, Morten B, Forbes JF, Braye SG, Avery-Kiejda KA, et al. Novel genes associated with lymph node metastasis in triple negative breast cancer. Scientific reports. 2015;5:15832.

26. Feber A, Guilhamon P, Lechner M, Fenton T, Wilson GA, Thirlwell C, et al. Using high-density DNA methylation arrays to profile copy number alterations. Genome Biol. 2014;15(2):R30.

27. Diskin SJ, Li M, Hou C, Yang S, Glessner J, Hakonarson H, et al. Adjustment of genomic waves in signal intensities from whole-genome SNP genotyping platforms. Nucleic Acids Res. 2008;36(19):e126-e.

28. Sapkota Y, Narasimhan A, Kumaran M, Sehrawat BS, Damaraju S. A Genome-Wide Association Study to Identify Potential Germline Copy Number Variants for Sporadic Breast Cancer Susceptibility. Cytogenetic Genome Research. 2016;149(3):156–64.

29. Lockwood WW, Chari R, Coe BP, Girard L, Macaulay C, Lam S, et al. DNA amplification is a ubiquitous mechanism of oncogene activation in lung and other cancers. Oncogene. 2008;27(33):4615–24.

30. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat. 2010;123(3):725–31.

31. Johnstone CN, Pattison AD, Gorringe KL, Harrison PF, Powell DR, Lock P, et al. Functional and genomic characterisation of a xenograft model system for the study of metastasis in triple-negative breast cancer. Dis Model Mech. 2018;11(5).

32. Engebraaten O, Vollan HKM, Borresen-Dale AL. Triple-negative breast cancer and the need for new therapeutic targets. Am J Pathol. 2013;183(4):1064–74.

33. Coussy F, de Koning L, Lavigne M, Bernard V, Ouine B, Boulai A, et al. A large collection of integrated genomically characterized patient-derived xenografts highlighting the heterogeneity of triple-negative breast cancer. Int J Cancer. 2019;145(7):1902–12.

34. Turner N, Lambros MB, Horlings HM, Pearson A, Sharpe R, Natrajan R, et al. Integrative molecular profiling of triple negative breast cancers identifies amplicon drivers and potential therapeutic targets. Oncogene. 2010;29(14):2013–23.

35. Goh JY, Feng M, Wang W, Oguz G, Yatim SMJM, Lee PL, et al. Chromosome 1q21.3 amplification is a trackable biomarker and actionable target for breast cancer recurrence. Nat Med. 2017;23(11):1319–30.

36. Gronwald J, Störkel S, Holtgreve-Grez H, Hadaczek P, Brinkschmidt C, Jauch A, et al. Comparison of DNA gains and losses in primary renal clear cell carcinomas and metastatic sites: importance of 1q and 3p copy number changes in metastatic events. Cancer Res. 1997;57(3):481–7.

37. Kjellman P, Lagercrantz S, Höög A, Wallin G, Larsson C, Zedenius J. Gain of 1q and loss of 9q21.3-q32 are associated with a less favorable prognosis in papillary thyroid carcinoma. Genes

Chromosomes Cancer. 2001;32(1):43–9.

38. Afshar-Kharghan V. The role of the complement system in cancer. J Clin Investig. 2017;127(3):780–9.

39. Shen Y, Yin R, Deng X, Shen H. Increased expression of CD55 correlates with tumor progression and poor prognosis in nasopharyngeal carcinoma. Clinical investigative medicine Medecine clinique et experimentale. 2012;35(1):E34-9.

40. Yin X, Shen C, Yin Y, Cai Z, Wang J, Zhao Z, et al. Overexpression of CD55 correlates with tumor progression and poor prognosis in gastric stromal tumors. Onco Targets Ther. 2019;12:4703–12.

41. Hirono S, Yamaue H, Hoshikawa Y, Ina S, Tani M, Kawai M, et al. Molecular markers associated with lymph node metastasis in pancreatic ductal adenocarcinoma by genome-wide expression profiling. Cancer Sci. 2010;101(1):259–66.

42. Qin LX, Tang ZY, Sham JS, Ma ZC, Ye SL, Zhou XD, et al. The association of chromosome 8p deletion and tumor metastasis in human hepatocellular carcinoma. Cancer Res. 1999;59(22):5662–5.

43. Thor AD, Eng C, Devries S, Paterakos M, Watkin WG, Edgerton S, et al. Invasive micropapillary carcinoma of the breast is associated with chromosome 8 abnormalities detected by comparative genomic hybridization. Hum Pathol. 2002;33(6):628–31.

44. Lebok P, Mittenzwei A, Kluth M, Özden C, Taskin B, Hussein K, et al. 8p deletion is strongly linked to poor prognosis in breast cancer. Cancer Biol Ther. 2015;16(7):1080–7.

45. Liu J, Miwa T, Hilliard B, Chen Y, Lambris JD, Wells AD, et al. The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. The Journal of experimental medicine. 2005;201(4):567–77.

46. Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP. Activation of human CD4 + cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature. 2003;421(6921):388–92.

47. Garner H, de Visser KE. Immune crosstalk in cancer progression and metastatic spread: a complex conversation. Nature Reviews Immunology. 2020.

48. Malin D, Chen F, Schiller C, Koblinski J, Cryns VL. Enhanced metastasis suppression by targeting TRAIL receptor 2 in a murine model of triple-negative breast cancer. Clin Cancer Res. 2011;17(15):5005–15.

49. Tanenbaum DG, Hall WA, Colbert LE, Bastien AJ, Brat DJ, Kong J, et al. TNFRSF10C copy number variation is associated with metastatic colorectal cancer. J Gastrointest Oncol. 2016;7(3):306–14.

50. Kiss T. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell. 2002;109(2):145–8.

51. Matera AG, Terns RM, Terns MP. Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. Nat Rev Mol Cell Biol. 2007;8(3):209–20.

52. Xu G, Yang F, Ding C-L, Zhao L-J, Ren H, Zhao P, et al. Small nucleolar RNA 113–1 suppresses tumorigenesis in hepatocellular carcinoma. Molecular Cancer. 2014;13(1):216.

53. Higgins J, Midgley C, Bergh A-M, Bell SM, Askham JM, Roberts E, et al. Human ASPM participates in spindle organisation, spindle orientation and cytokinesis. BMC Cell Biol. 2010;11(1):85.

54. Riggs KA, Hasan N, Humphrey D, Raleigh C, Nevitt C, Corbin D, et al. Regulation of integrin endocytic recycling and chemotactic cell migration by syntaxin 6 and VAMP3 interaction. J Cell Sci. 2012;125(Pt 16):3827–39.

55. Lin SY, Pan HW, Liu SH, Jeng YM, Hu FC, Peng SY, et al. ASPM is a novel marker for vascular invasion, early recurrence, and poor prognosis of hepatocellular carcinoma. Clin Cancer Res. 2008;14(15):4814–20.

56. Bikeye SN, Colin C, Marie Y, Vampouille R, Ravassard P, Rousseau A, et al. ASPM-associated stem cell proliferation is involved in malignant progression of gliomas and constitutes an attractive therapeutic target. Cancer Cell Int. 2010;10:1.

57. Brüning-Richardson A, Bond J, Alsiary R, Richardson J, Cairns DA, McCormack L, et al. ASPM and microcephalin expression in epithelial ovarian cancer correlates with tumour grade and survival. Br J Cancer. 2011;104(10):1602–10.

58. Xie J-J, Zhuo Y-J, Zheng Y, Mo R-J, Liu Z-Z, Li B-W, et al. High expression of ASPM correlates with tumor progression and predicts poor outcome in patients with prostate cancer. Int Urol Nephrol. 2017;49(5):817–23.

59. Wang WY, Hsu CC, Wang TY, Li CR, Hou YC, Chu JM, et al. A Gene Expression Signature of Epithelial Tubulogenesis and a Role for ASPM in Pancreatic Tumor Progression. Gastroenterology. 2013;145(5):1110–20.

60. Corson TW, Gallie BL. KIF14 mRNA expression is a predictor of grade and outcome in breast cancer. Int J Cancer. 2006;119(5):1088–94.

61. Corson TW, Huang A, Tsao MS, Gallie BL. KIF14 is a candidate oncogene in the 1q minimal region of genomic gain in multiple cancers. Oncogene. 2005;24(30):4741–53.

62. Zhang Y, Yuan Y, Liang P, Zhang Z, Guo X, Xia L, et al. Overexpression of a novel candidate oncogene KIF14 correlates with tumor progression and poor prognosis in prostate cancer. Oncotarget. 2017;8(28):45459–69.

63. Thériault BL, Pajovic S, Bernardini MQ, Shaw PA, Gallie BL. Kinesin family member 14: an independent prognostic marker and potential therapeutic target for ovarian cancer. Int J Cancer. 2012;130(8):1844–54.

64. Xu H, Choe C, Shin SH, Park SW, Kim HS, Jung SH, et al. Silencing of KIF14 interferes with cell cycle progression and cytokinesis by blocking the p27(Kip1) ubiquitination pathway in hepatocellular carcinoma. Exp Mol Med. 2014;46(5):e97.

65. Sasahira T, Kurihara M, Nakashima C, Kirita T, Kuniyasu H. LEM domain containing 1 promotes oral squamous cell carcinoma invasion and endothelial transmigration. Br J Cancer. 2016;115(1):52–8.

66. Li Q, Ge Y, Chen X, Wang L, Xia Y, Xu Z, et al. LEM domain containing 1 promotes proliferation via activating the PI3K/Akt signaling pathway in gastric cancer. J Cell Biochem. 2019;120(9):15190–201.

67. Vazquez-Mena O, Medina-Martinez I, Juárez-Torres E, Barrón V, Espinosa A, Villegas-Sepulveda N, et al. Amplified Genes May Be Overexpressed, Unchanged, or Downregulated in Cervical Cancer Cell Lines. PLOS ONE. 2012;7(3):e32667.

9. Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.
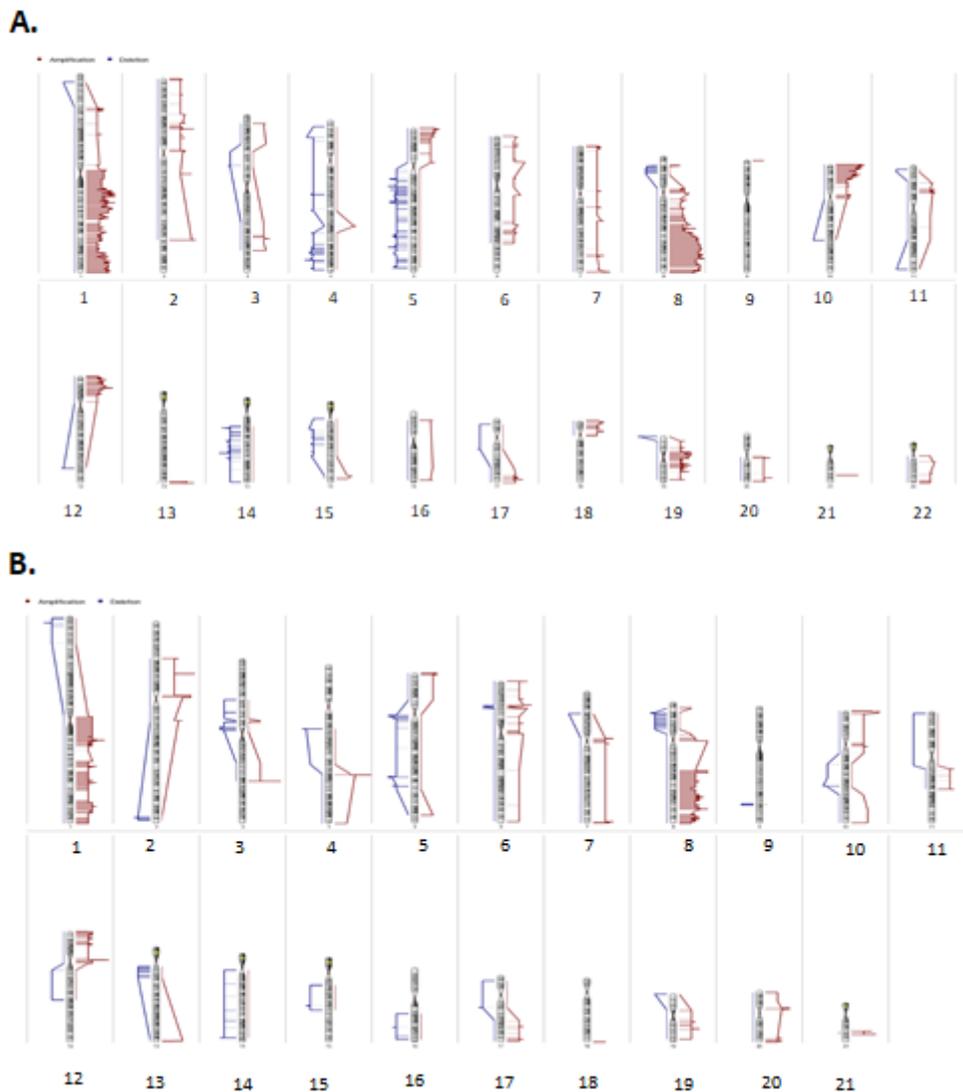
Figures

Figure 1

Karyogram view of significantly amplified (red) and deleted (blue) regions across 22 chromosomes of the study cohort containing 23 IDCs (Top) and 12 LNmets (below). Histogram heights on either side of the chromosome correspond to the number of samples that share either amplification or deletion at the

particular region. The higher the histogram height, the higher the number of samples amplified or deleted at that particular region. This karyogram profile was generated using a genomic segmentation algorithm with the number of minimum genomic markers >=40, p-value=0.001 and signal to noise ratio=0.3. A false discovery of 0.05 was applied to the resulting p-values to correct for multiple testing. A. CNV profile in IDC samples where amplification in 8q, 1q, 2q, 10p and 12p is shared by >50% of samples. Deletion in 4q, 5q, 14q and 19p is shared by >39% of samples. B. CNV profile in LNmet samples where the amplification in 1q, 4q28.3, 2p, 3q, 6p, 8q, 12p, 20p and 21p is shared by >50% of samples, whereas the deletion in 1p, 4q and 5q is shared by >39% of samples. .
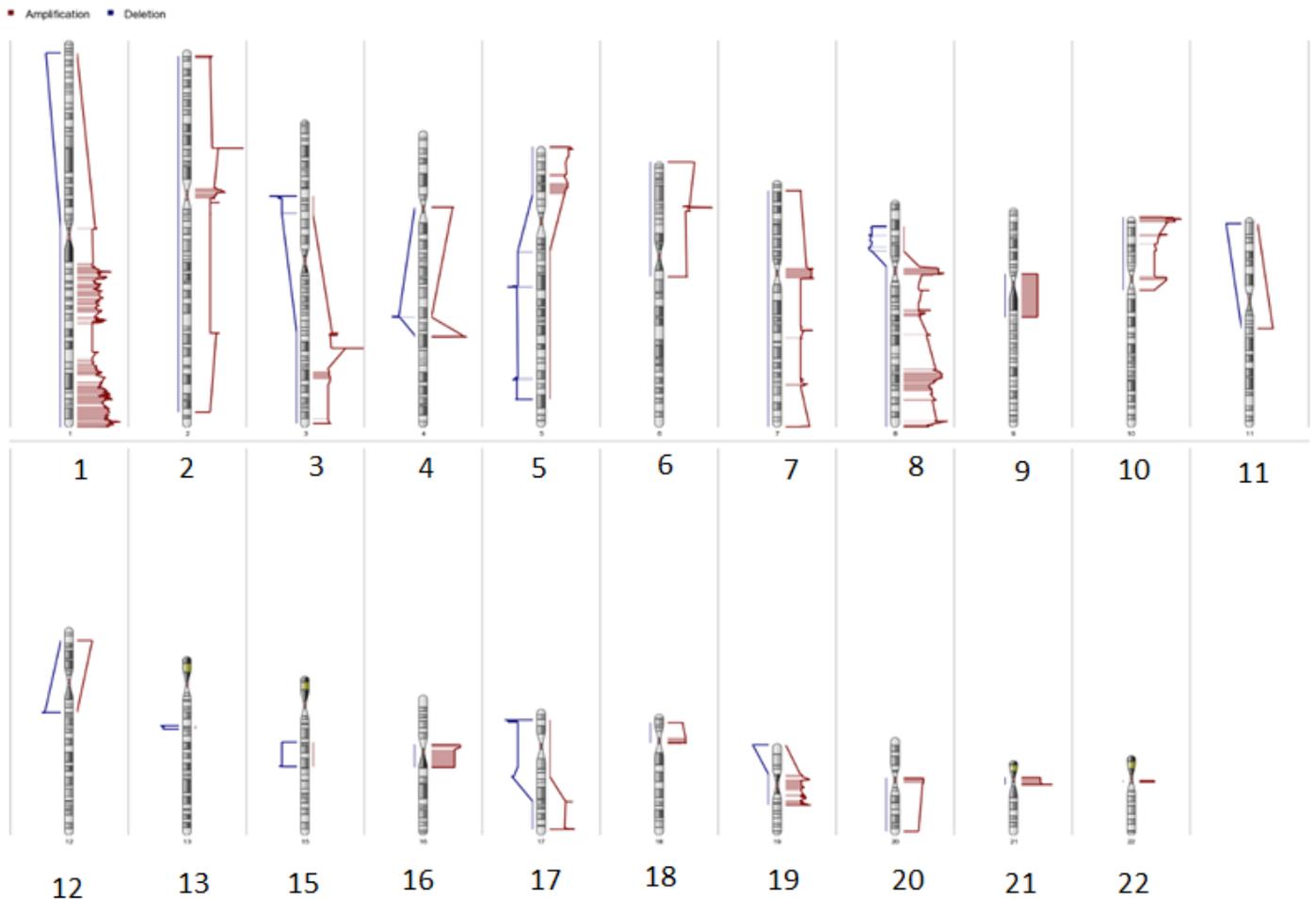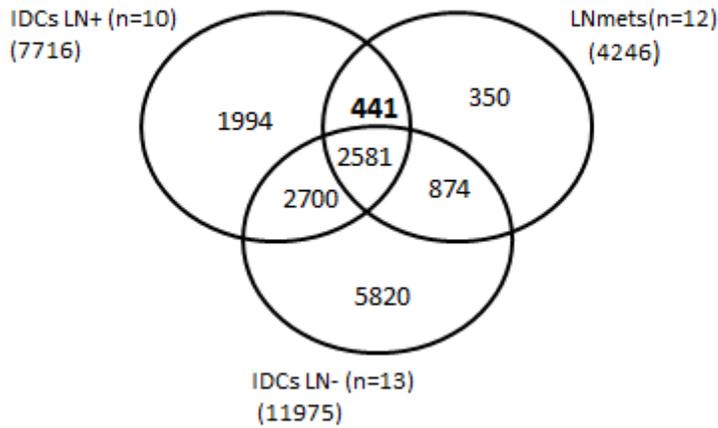

Figure 2

Karyogram view of significantly amplified (red) and deleted (blue) regions across 21 chromosomes of 70 IDCs in the second cohort. Histogram heights on either side of the chromosome corresponds to the number of samples that share either amplification or deletion at the particular region. The higher the histogram height the higher the number of samples that are amplified or deleted at that particular region. This karyogram profile was generated using a genomic segmentation algorithm with the number of minimum genomic markers >=40, p-value=0.001 and a signal to noise ratio=0.3. A false discovery of 0.05 was applied to the resulting p-values for multiple testing. CNV profile in IDCs sample where amplification

in 3q, 2p, 6p, 8q, 1q, 10p, 4q is shared by >50% of samples. Deletion in 17p and 3p is shared by >39% of samples.
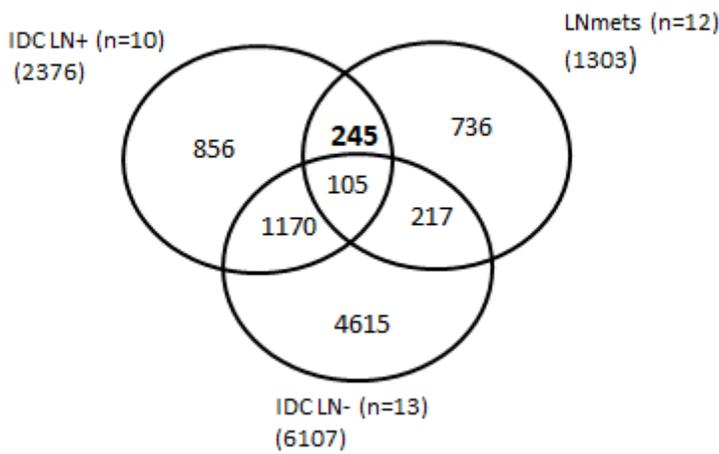
A. Amplification

B. Deletion

Figure 3

Venn diagram showing genes associated with lymph node metastasis. The gene lists were identified by overlapping the significant CNV regions that are shared by at least 22% of multiple samples. The number of genes for: IDC Lymph node positive (IDC LN+), IDC Lymph node negative (IDC LN-) and LNmets are shown in brackets both for amplification and for deletion and n refers to number of samples in each group. A. 441 amplified genes highlighted in bold were common in both IDC LN+ and LNmets. B. 245 deleted genes highlighted in bold were common in both IDC LN+ and LNmets.
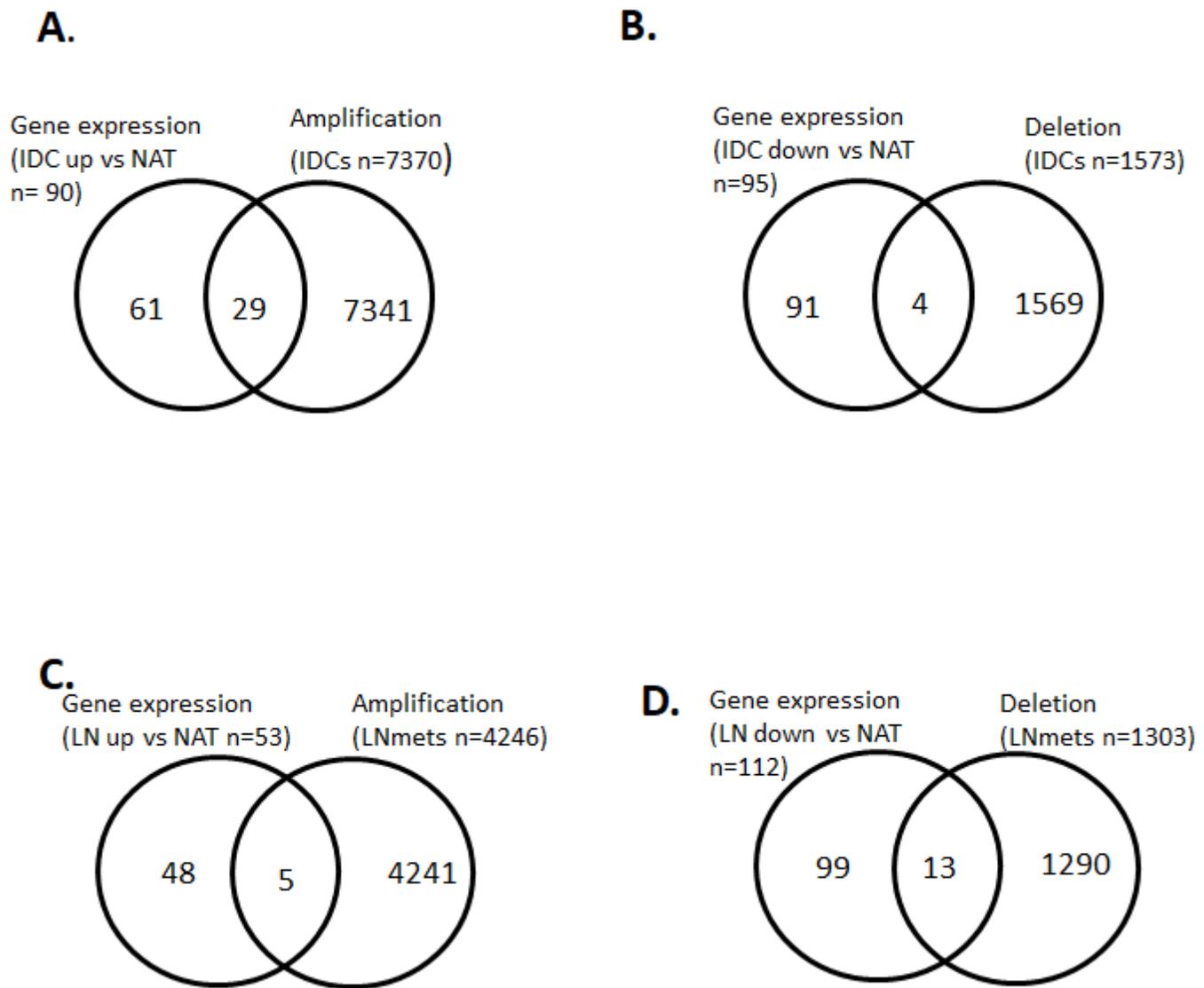
A.

Gene expression
(IDC up vs NAT
n= 90)

Amplification
(IDCs n=7370)

61 | 29 | 7341

B.

Gene expression
(IDC down vs NAT
n=95)

Deletion
(IDCs n=1573)

91 | 4 | 1569

C.

Gene expression
(LN up vs NAT n=53)

Amplification
(LNmets n=4246)

48 | 5 | 4241

D.

Gene expression
(LN down vs NAT
n=112)

Deletion
(LNmets n=1303)

99 | 13 | 1290

Figure 4

Venn diagram showing genes with CNV and change in gene expression. Total 33 of 185 (18%) differentially expressed genes in IDC vs NAT were also CNV altered in the IDCs of study cohort where A. 29 upregulated genes were amplified and B. 4 downregulated genes were deleted. Total 18 of 165 (10.9%) differentially expressed genes in LN vs NAT were also CNV altered in LNmets of study cohort where C. 5 upregulated genes were amplified and D. 13 downregulated genes deleted.

a. ASPM

HR = 1.73 (1 - 2.99)
logrank P = 0.047

Expression
— low
— high

Number at risk

low	89	47	16	2	0
high	72	27	4	1	0

b. KIF14

HR = 1.77 (0.99 - 3.17)
logrank P = 0.05

Expression
— low
— high

Number at risk

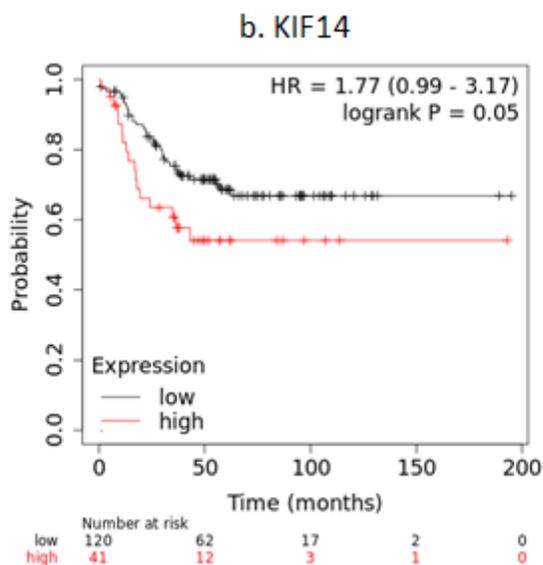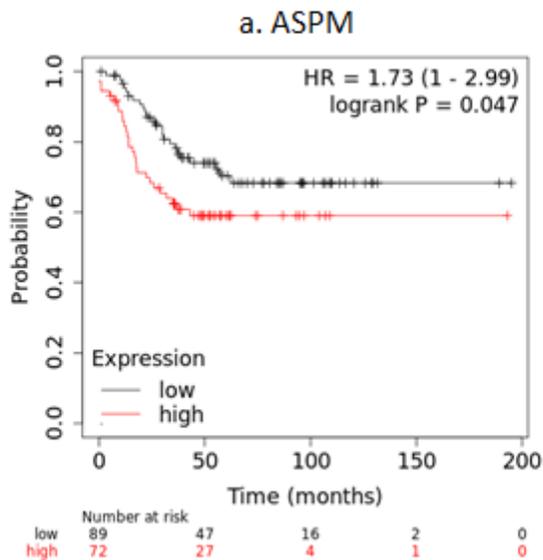|				
low	120	62	17	2	0
high	41	12	3	1	0

Figure 5

Kaplan Meier plots for relapse free survival curves of the patients with high expression (red) and low expression (black) for a. ASPM and b.KIF14. High expression of both ASPM and KIF14 were significantly associated with reduced relapse free survival. https://kmplot.com/analysis/index.php?p=service&cancer=breast

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile12TableS15S16.xlsx
- Additionalfile11TableS14.xlsx

- Additionalfile10TableS13.xlsx
- Additionalfile9TableS12.xlsx
- Additionalfile8TableS11.xlsx
- Additionalfile7TableS9S10.xlsx
- Additionalfile6TableS8.xlsx
- Additionalfile5TableS7.xlsx
- Additionalfile4TableS6.xlsx
- Table1.pptx
- Additionalfile3TableS5.xlsx
- Additionalfile2TableS4.xlsx
- Additionalfile1TableS1S2S3.xlsx