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Integrative molecular profiling of triple negative breast cancers identifies amplicon drivers and potential therapeutic targets

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Abstract

Triple negative breast cancers (TNBCs) have a relatively poor prognosis and cannot be effectively treated with current targeted therapies. We searched for genes that have the potential to be therapeutic targets by identifying genes consistently over-expressed when amplified. Fifty-six TNBCs were subjected to high-resolution microarray-based comparative genomic hybridisation (aCGH), of which 24 were subjected to genome-wide gene expression analysis. TNBCs were genetically heterogeneous; no individual focal amplification was present at high frequency, although 78.6% of TNBCs harboured at least one focal amplification. Integration of aCGH and expression data revealed 40 genes significantly overexpressed when amplified, including the known oncogenes and potential therapeutic targets, *FGFR2* (10q26.3), *BUB3* (10q26.3), *RAB20* (13q34), *PKN1* (19p13.12), and *NOTCH3* (19p13.12). We identified two TNBC cell lines with *FGFR2* amplification, which both had constitutive activation of FGFR2. Amplified cell lines were highly sensitive to FGFR inhibitor PD173074, and to RNAi silencing of *FGFR2*. Treatment with PD173074 induced apoptosis resulting partly from inhibition of PI3K-AKT signalling. Independent validation using publicly available aCGH datasets revealed *FGFR2* gene was amplified in 4% (6/165) of TNBC, but not in other subtypes (0/214, p=0.0065). Our analysis demonstrates that TNBCs are heterogeneous tumours with amplifications of FGFR2 in a subgroup of tumours.

Keywords

Triple negative breast cancer; microarrays; gene expression; comparative genomic hybridisation; FGFR2

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Introduction

The identification of distinct subgroups of breast cancer has led to the development of therapeutic strategies that exploit the underlying biology of the subtype, with hormonal therapies and HER2 targeting agents for hormone receptor-positive and HER2-positive breast cancers, respectively (Reis-Filho and Tutt, 2008; Schneider *et al.*, 2008). Tumours lacking expression of hormone receptors (oestrogen receptor (ER) and progesterone receptor (PR)) and HER2 (triple negative breast cancers, TNBCs), on the other hand, pose a significant clinical challenge due to a poor understanding of the genetic alternations that underlie the development of TNBC (Reis-Filho and Tutt, 2008; Schneider *et al.*, 2008) and this is reflected in a lack of subtype-specific targeted therapies.

TNBCs comprise a heterogeneous group of breast cancers and account for 10-15% (Agrawal *et al.*, 2007; Carey *et al.*, 2007; Dent *et al.*, 2007; Reis-Filho and Tutt, 2008; Schneider *et al.*, 2008) of all invasive breast cancers. Histologically, the majority of TNBCs are grade III invasive ductal carcinomas of no special type (IDC-NST), although the majority of medullary, metaplastic and adenoid cystic carcinomas also display a triple negative phenotype (Reis-Filho and Tutt, 2008). These tumours are more prevalent in young women (<50 years) and in women of African and Hispanic descent. TNBCs have a poor prognosis characterised by early relapse (Dent *et al.*, 2008; Tischkowitz and Foulkes, 2006), potentially reflecting the high proliferative rate of TNBCs. Similarly, women with TNBCs have a significantly shorter survival following recurrence when compared to those with non-triple negative cancers (Dent et al., 2007; Harris et al., 2006). Therefore, the identification of novel therapeutic targets for TNBCs is important if the outcome of patients with these tumours is to be improved.

Based on the concept of oncogene addiction, we and others have demonstrated that genes that are consistently overexpressed when amplified may be selectively required for the survival of cancer cells harbouring their amplification, and can be exploited as potential therapeutic targets (Bernard-Pierrot et al., 2008; Natrajan et al., 2009a; Reis-Filho et al., 2006). Previous studies have examined TNBCs with expression profiling (Bertucci et al., 2008; Kreike et al., 2007) and microarray-based comparative genomic hybridisation (aCGH) (Andre et al., 2009; Han et al., 2008). These studies have found TNBCs to be heterogeneous, with complex genomic profiles and infrequent amplifications.

To identify amplicon drivers and genes that have the potential to be therapeutic targets in TNBCs, we integrated aCGH and gene expression data from a large series of TNBCs. Our aims were to characterise the genomic and transcriptomic profiles of TNBCs and identify and validate genes that are recurrently amplified and consistently overexpressed when amplified in TNBCs. We found more frequent high level, focal amplifications than previously described (Chin et al., 2006), and identified potential therapeutic targets that are consistently overexpressed when amplified. We validated the fibroblast growth factor receptor 2 (*FGFR2*) gene as one of these targets and provided functional data to suggest that this tyrosine kinase receptor may be a novel therapeutic target in a subset of TNBCs harbouring *FGFR2* gene amplification.

Material and Methods

Tumour samples

Fifty-six fresh-frozen samples of TNBCs were obtained after approval by local Ethic Committees from the authors' institutions. Triple negative tumours were selected according to their lack of expression to ER, PR and HER2 as defined by immunohistochemistry (Kreike et al., 2007). All tumours were morphologically invasive ductal cancers of no

special type. ER, PR and HER2 antibodies, antigen retrieval systems and scoring methods are summarised in Supplementary Table 1. One representative section of each tumour was stained with haematoxiylin-eosin. Samples were either microdissected with a sterile needle under a stereomicroscope (Marchio *et al.*, 2008), or samples from Kreike et al. (Kreike et al., 2007) were only included if there were >70% of neoplastic cells in the section. A complete description of the cohort analysed here is described in Supplementary Table 2. Out of the samples included in this study, the aCGH profiles of 23 cases were reported in Natrajan et al. (Natrajan et al., 2009a) and the expression profiles of 24 cases were reported in Kreike et al. (Kreike et al., 2007). No statistically significant differences were observed in terms of patient age, tumour size, histological grade, prevalence of basal-like phenotype and outcome between the 24 cases included in this study, and the remaining TNBCs from Kreike et al. (2007; Supplementary Table 3).

RNA and DNA extraction

DNA and RNA were extracted as previously described. DNA concentration was measured with Picogreen® (Invitrogen, Paisley UK) according to the manufacturer's instructions (Marchio *et al.*, 2008).

Microarray-Based Comparative Genomic Hybridisation

The 32K BAC re-array collection (CHORI) tiling path aCGH platform was constructed at the Breakthrough Breast Cancer Research Centre, as described previously (Marchio *et al.*, 2008).This type of BAC array platform has been shown to be as robust as and to have comparable resolution with high density oligonucleotide arrays (Coe et al., 2007; Gunnarsson et al., 2008; Tan et al., 2007). DNA labelling, array hybridisations and image acquisition were performed as previously described (Natrajan et al., 2009a). aCGH data were pre-processed and analysed using an in-house R script (BACE.R) in R version 2.9.0, as previously described (Mackay et al., 2009; Natrajan et al., 2009b). After filtering polymorphic BACs, a final dataset of 31544 clones with unambiguous mapping information according to the August 2009 build (hg19) of the human genome (http://www.ensembl.org) was smoothed using the circular binary segmentation (cbs) algorithm (Mackay et al., 2009; Natrajan et al., 2009b). A categorical analysis was applied to the BACs after classifying them as representing amplification (>0.45), gain (>0.08 and ≤0.045), loss (<−0.08), or no-change according to their cbs-smoothed Log_2 ratio values (Natrajan et al., 2009b; Reis-Filho et al., 2008). Threshold values were determined and validated as previously described (Natrajan et al., 2009b).

Tumours were classified according to their pattern of genomic alterations into 'simplex', 'firestorm' or 'sawtooth' as previously described(Hicks et al., 2006; Natrajan et al., 2009a).

Gene expression analysis

RNA labelling, hybridisation, slide scanning and data normalisation were performed as previously described (Kreike et al., 2007; Weigelt et al., 2008). Fluorescent intensities were normalised and corrected for biases (Hannemann et al., 2006), and weighted averages and confidence levels were computed according to the Rosetta Error Model (Hughes et al., 2000). All the expression data can be retrieved from http://www.ebi.ac.uk/microarrayas/ae/browse.html?keywords=E-NCMF-2&species=&array=&exptype=&pagesize=25&sortby=releasedate&sortorder=descending. Of the TNBCs analysed, 94.2% were of basal-like phenotype using the criteria described by Hu et al. (Hu et al., 2006) (Supplementary Table 2).

Identification of genes whose expression correlates with copy number changes

To identify genes whose expression levels correlate with copy number changes, cbs-smoothed Log_2 ratios from aCGH data were used to assign the aCGH states for each of the 24,650 genes in the gene expression dataset using the median values for all BACs which overlap with the genomic position of each gene. This resulted in a 1:1 matrix of expression ratios and aCGH cbs values, which were used for downstream statistical analysis. Pearson's correlations were performed between gene expression array Log_2 ratios and cbs-smoothed ratios derived from aCGH analysis for each gene. The p values for each test were adjusted with Benjamini and Hochberg multiple p-value adjustment (Benjamini and Hochberg, 1995). Adjusted p values < 0.05 were considered significant.

To define genes that were upregulated when gained, downregulated when lost or overexpressed when amplified, we performed a multi-Mann–Whitney U test using categorical aCGH states (i.e. gain vs no gain, loss vs no loss or amplification vs no amplification) as the grouping variable and the expression of genes as the dependent variable as previously described (Mackay et al., 2009; Natrajan et al., 2009b). P-values were adjusted using Benjamini and Hochberg multiple comparison p-value adjustment (Benjamini and Hochberg, 1995). Adjusted p values < 0.05 were considered significant.

For regions of recurrent amplification, matched heatmaps were created by retrieving gene expression values and corresponding median-overlay aCGH states for each gene as previously described (Mackay et al., 2009; Natrajan et al., 2009b). Genes were ordered according to chromosomal location and cases were separated into those that harbour amplifications in the region and those which do not. Within these groups the samples were ordered based upon the sum of expression values within the region.

Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) program (http://www.ingenuity.com) was used as previously described (Natrajan et al., 2009b). HUGO gene identifiers were mapped to networks available in the Ingenuity database and ranked by score. The score indicates the likelihood of the genes in a network being found together due to random chance. Using a 99% confidence level, scores of ≥ 3 are considered significant.

Cell lines, materials and antibodies

Cell lines were obtained from ATCC or DMSZ, and maintained in DMEM or RPMI with 10% FBS (PAA gold) and 2mM L-glutamine (Sigma) (details available on request). S68 was a kind gift of Veronique Catros, Rennes, France. PD173074 was obtained from Sigma, BEZ235 from Axon Medchemicals, and U0126 from Calbiochem. siRNA were purchased from Dharmacon: FGFR2 siGenome SMARTpool (siFGFR2, M-003132-04) and siGENOME Non-Targeting siRNA Pool #1 (siCON, D-001206-13). Antibodies used were FGFR2 (sc-122, Santa-Cruz), phospho-FRS2-Tyr196 (3864, Cell Signaling), phospho-AKT-Ser473 (4058, Cell Signaling), phospho-ERK1/2-Thr202/Tyr204 (4370, Cell Signaling), and β-Actin (sc-1616, Santa-Cruz).

Cell line drug sensitivity, siRNA transfection and FACS analysis

Cell lines were transfected with siCON or siFGFR2 in 96 well plates with Lipofectamine RNAiMax (Invitrogen), Lipofectamine 2000 (Invitrogen), Dharmafect 3 (Thermo Scientific), or Oligofectamine (Invitrogen) according to manufacturers instructions. Transfection of all cell lines was confirmed with positive control PLK1 siRNA (loss of survival $< 25\%$ that of siCON transfection in all cell lines, Supplementary Figure 3). Survival was assessed with Cell Titre-Glo® cell viability assay (Promega) after five population doublings or 7 days which ever was shorter. For sensitivity to PD173074, cell

lines were plated in 96 well plates, the following day media supplemented with PD173074 at increasing concentrations, and survival assessed with Cell Titre-Glo® after 96 hours exposure. Survival curves and estimated SF50 were calculated with GraphPad prism V5.0. For Combination Index (CI) cells were treated in 96 well plate for 96 hrs with two fold dilutions of PD173074, BEZ235, or combination, and CI calculated using non-mutually exclusive median effect model as described previously (Chou and Talalay, 1984). FACS analysis was performed as described previously (Turner et al., 2008).

Western blotting

Indicated cell lines where grown on 10cm plates, and grown for 24 hrs in serum free medium or normal medium. Where indicated plates were treated with drug for 1 hour prior to lysis. Western blots were carried out with precast TA or Bis-Tris gels (Invitrogen) as described previously (Turner et al., 2008).

Fluorescence in situ hybridisation for FGFR2

FGFR2 gene copy number status was assessed in breast cancer cell lines with in-house generated BAC probes comprising two BACs (RP11-300A10 and RP11-753P11), which map to specifically to the *FGFR2* gene locus, as previously described (Lambros et al., 2006).

Results

TNBCs display complex genomic profiles

We established the genomic array CGH profiles of 56 TNBCs with an array CGH platform with a previously validated effective resolution of 50Kb. This revealed a high level of genetic instability, with gain or loss affecting a median of 44.4% of the genome (range 9.3%-76.7%). Using the genomic pattern classification proposed by Hicks et al. (2006) 15 of the TNBCs (27%) were classified as ‘simplex’ and 41 (73%) were considered to have ‘complex’ genomic patterns, of which 28 (50%) were ‘sawtooth’ and 13 (23%) were ‘firestorm’ (Hicks et al., 2006). Given that the majority of TNBCs displayed ‘sawtooth’ patterns, it is not surprising that multiple regions of recurrent gains and losses were identified (Figure 1a and Supplementary Table 4). In agreement with previous studies (Andre et al., 2009; Han et al., 2008), loss of 1p36-p34, 5q11-q35, 8p23.3-p12 and 17p13-q21 and gain of 3q22-q26, 6p25-p21, 7q32-q36, 8p11-q24, 10p15.3-p11.21 and 12p13-p11 were found in >30% of TNBCs (Figure 1a). Recurrent high level gains and amplifications were found in TNBCs (Figure 1b and Supplementary Table 5). At least one focal amplification (<10Mb) was found in 78.6% (44/56) of cases; however, the prevalence of each amplification was relatively low, ranging from 4% to 27% (Supplementary Table 5).

Integrative aCGH and expression analysis reveal pathways and networks that are enriched for genes whose expression correlates with copy number

To determine the genes whose expression levels correlate with copy number, we overlaid aCGH and expression data in an unbiased, genome-wide fashion (Natrajan et al., 2009b). This analysis revealed that the expression of 4972 out of 24565 genes (20.2%) correlated with copy number changes (Pearson’s correlation adjusted $p<0.05$, Supplementary Table 5). This analysis suggests that a large proportion of the genes expressed in TNBCs are at least in part regulated by copy number changes.

Within this list we determined the genes whose expression was significantly upregulated or downregulated in the presence of copy number gain or loss respectively (Mann-Whitney U adjusted $p<0.05$, Supplementary Table 6). We identified 324 genes whose mRNA expression levels were significantly higher in tumours harbouring DNA copy number gains and 39 genes whose mRNA expression levels were significantly lower in tumours

displaying DNA copy number loss. The lower number of genes down-regulated when lost may stem from the slightly lower sensitivity of array CGH to detect loss of a single copy of a genomic region in highly aneuploid tumours (Ng et al., 2006), coupled with the limitations of expression arrays to accurately determine the expression of genes expressed at low levels.

Ingenuity pathway analysis (IPA) of genes expressed at higher levels when gained revealed that 5 networks and 27 canonical pathways were significantly enriched (Supplementary Table 8). Importantly, the canonical pathways of growth factors that have been shown to play a role in breast cancer development and progression, or to be potential therapeutic targets, were significantly enriched with genes upregulated when gained (Table 1). Copy number gains of the tyrosine kinase receptors were rare, and the growth factor pathway enrichment reflected frequent copy number gains and over-expression of key adapter molecules and down-stream signal transduction kinases (Table 1). The p53 signalling pathway was enriched for genes that are upregulated when gained, including *survivin* (*BIRC5*).

Recurrent amplified regions and potential amplicon drivers

We next analysed the data to identify the genes that were significantly overexpressed when amplified in TNBCs, compared to non-amplified cancers. This analysis revealed only 40 genes (Mann-Whitney U test adjusted $p<0.05$, Table 2) but included multiple genes that have been shown to either have oncogenic properties or to be potential therapeutic targets, such as *FGFR2* (10q26), *BUB3* (10q26), *RAB20* (13q34), *NOTCH3* (19p13) and *PKN1* (19p13). It should be noted that this analysis, with a non-parametric rank sum test corrected for false discovery, is intentionally conservative and will identify genes that are robustly over-expressed when amplified. Such an analysis is not intended to be exhaustive. Of the 40 genes, 38 also displayed expression levels that correlated with copy number as assessed by Pearson correlation (Supplementary Table 10). Genes that are robustly over-expressed when amplified potential amplicon driver, and therapeutic targets, as has been suggested for *NOTCH3* (Yamaguchi et al., 2008), and *BUB3* (Yuan et al., 2006) (Table 2).

FGFR2 is amplified in triple negative breast cancers and breast cancer cell lines

We selected, for further investigation, *FGFR2* amplifications from the list amplified and over-expressed, as there are a number of drugs in early phase clinical trials that target the FGFRs. A re-analysis of data from publicly available aCGH datasets (Adelaide et al., 2007; Andre et al., 2009; Chin et al., 2006), including our current data set, revealed *FGFR2* amplification in 4% (6/165 95% CI 1.4-7.8%) of TNBCs, with no cases of *FGFR2* amplification in other subtypes (0/214, p=0.0065. Fisher's exact test). Excluding our data set, the frequency was similar (TNBC 4% (4/109) vs other subtypes 0% (0/214), p=0.0125).

We screened a panel of 51 breast cancer cell lines using a combination of aCGH and western blotting of cell lysates to identify cell lines with *FGFR2* gene amplification and protein overexpression. We identified two cell lines with *FGFR2* amplification one of which, SUM52PE, had been described previously (Tannheimer et al., 2000). MFM223 was found to express high levels of FGFR2 protein (Figure 3a) and to harbour *FGFR2* gene amplification by aCGH (Figure 3b). *FGFR2* amplification was confirmed by *FGFR2* FISH in MFM223 (Figure 3c), and by copy number PCR (Supplementary Figure 2). Both amplified cell lines expressed substantially higher *FGFR2* mRNA than all other cell lines (SUM52PE 67 fold and MFM223 26 fold higher than median expression of non-amplified lines, Supplementary Figure 1). Both MFM223 and SUM52 were found to be triple negative, with neither cell line expressing ER or PR by western blotting nor harbouring *HER2* gene amplification (Supplementary Figure 1).

FGFR2 expression and tyrosine kinase activity are required for the survival of cell lines with FGFR2 gene amplification

We transfected a panel of cell lines with *FGFR2* siRNA (Figure 3d). FGFR2 silencing selectively reduced the survival of MFM223 cells, *FGFR2* amplified cell line, compared to all transfectable non-amplified cell lines (Figure 3, and Supplementary Figure 3). Silencing of FGFR2 by siRNA has confirmed by western blot (Supplementary Figure 3). We then examined the sensitivity of the cell line panel to PD173074, a highly potent selective pan-FGFR tyrosine kinase inhibitor (Mohammadi et al., 1998). The *FGFR2* amplified cell lines were substantially more sensitive to PD173074 than all comparator cell lines (Figure 3e), indicating that FGFR kinase activity was selectively required for the growth/survival of cell lines harbouring *FGFR2* gene amplification.

Ligand independent signalling in FGFR2 amplified cell lines

Signalling through the FGFR family of growth receptors is reliant on the adapter protein Fibroblast Receptor Substrate 2 alpha (FRS2), which binds directly to phosphorylated FGFR forming a FRS2-SOS-GRB2 complex to activate MAPK signalling and a FRS2-SOS-GAB1 complex to activate PI3K-AKT signalling. We examined the signal transduction pathways activated downstream of FGFR2 in serum starved conditions with and without PD173074 to inhibit FGFR2 kinase activity. FRS2 exhibits a mobility shift, reflecting phosphorylation, in serum starved amplified cell lines, which is abolished by treatment with PD173074, indicating ligand independent constitutive activation of the receptor (Figure 4a). In both cell lines harbouring *FGFR2* gene amplification, AKT Ser473 was phosphorylated in an FGFR kinase dependent manner, with ERK1/2 Thr202/Tyr204 phosphorylated in an FGFR kinase dependent fashion in MFM223. SUM52PE expressed low levels of phosphorylated ERK, which presumably was reflected in the low proliferative rate of this cell line (data not shown). Phosphorylation of AKT and ERK1/2 was independent of FGFR kinase activity in control cell lines MCF7, SKBR3 and HCC1143 (Figure 4).

FGFR2 amplified MFM223 die by apoptosis following FGFR inhibition

We investigated the mechanism of loss of survival in MFM223 on PD173074 treatment. After 48 hr treatment with PD173074 there was no significant alteration of the cell cycle profile of the MCF7 control cell line, but there was a substantial increase in subG1 cells in MFM223 (Figure 5). To investigate whether the increase in subG1 reflected increased apoptosis, we assessed Annexin V/PI staining in MCF7 and MFM223 cells treated for 48hrs with PD173074 (Figure 5b). There was no difference in the proportion of apoptotic, Annexin V positive/PI negative, cells in MCF7 without and with PD173074 (4.8% vs 3.5%, p=NS), but a substantial increase in apoptotic cells in MFM223 (2.3% vs 11.4% respectively, $p<0.001$).

To confirm that these observations represented ligand independent signalling, we grew MFM223 in serum free medium. In the absence of serum MFM223 proliferated at a similar rate compared to cells grown in 10% serum. In the absence of serum MFM223 were dependent on FGFR for proliferation, and underwent apoptosis in the presence of PD173074 (Supplementary Figure 4).

FGFR2 amplified cell lines also have PI3 kinase pathway aberrations

We noted that AKT was highly phosphorylated in MFM223, but that this was substantially decreased in PD173074 treated cells. To investigate whether this reflected an activating mutation in *PIK3CA*, we sequenced the helical and kinase domains of *PIK3CA* and identified a classical H1047R kinase mutation in MFM223 (Supplementary Figure 5). The H1047R mutation has been described to active PI3 kinase function and transforms human

mammary epithelial cells (Zhao *et al.*, 2005). We therefore examined the effect of dual targeting of PI3 kinase and FGFR. Treatment of MFM223 with the dual PI3 kinase/mTOR inhibitor BEZ235 (Maira et al., 2008) abolished AKT phosphorylation (Supplementary Figure 5) and substantially increased the subG1 fraction (Figure 5c). Treatment with combination of BEZ235 and PD173074 increased the subG1 fraction to a greater extent than either drug given individually (Figure 5c and Supplementary Figure 2). Similarly, dual treatment with both inhibitors had an additive effect as assessed by combination index (CI) assay (Supplementary Figure 5, CI 1.02). SUM52 had wild-type *PIK3CA*, but did not express PTEN protein (Supplementary Figure 5), and a similar additive CI was observed in SUM52 cells between PD173074 and BEZ235 (CI 0.985). These data suggests that dual targeting of FGFR2 and PIK3CA may be of benefit in cancers with *FGFR2* amplification.

Taken together, our data provide strong circumstantial evidence that FGFR2 may be a therapeutic target for a subset of TNBC harbouring *FGFR2* gene amplification, and that our method of integrated analysis of transcriptomic and genomic data, focused on genes that are over-expressed when amplified, identifies potential treatment targets.

Discussion

TNBCs are genetically unstable and often harbour complex patterns of genetic aberrations. Using high resolution array CGH and gene expression platforms, tumours with >70% of neoplastic cells, and only invasive ductal cancers of no special type, we have demonstrated that a substantial proportion of genes have expression levels that significantly correlate with copy number in TN cancers. Functional annotation of these genes using IPA revealed that the canonical pathways of several tyrosine kinase receptors involved in tumourigenesis and cancer progression were enriched for the genes upregulated by copy number gain were enriched (Supplementary Table 8). Interestingly, copy number gains of the tyrosine kinase receptors were rare; instead, upregulation of signal transduction kinases downstream of the receptors (e.g. RAF1, PTK2, PIK3C2G, CSNK1D, MAPK9) and adaptor proteins (e.g., GRB2, GAB1) was observed. It is possible that these recurrent copy number gains, of multiple components of the canonical pathways of tyrosine kinase receptors, could create a permissive context for activation of these pathways in TN breast cancers. In addition this observation suggests that in TNBCs it may be possible to identify commonly activated signal transduction pathways that could be targeted effectively for TNBC therapy.

The majority of TNBCs showed losses on 1p, 2q, 3p, 4p, 5q, 8p 9q, 16q, 17p, 19p, and 23p; and gains on 1p, 3q, 6p, 9p, 7q, 8p, 10p, and 12p. Only 11% of the tumours in our cohort showed concurrent 1q gain and 16q loss, the typical changes of low-grade ER positive breast cancers, in agreement with our previous results suggesting that progression from grade I to grade III is an uncommon phenomenon in TNBCs (Natrajan et al., 2009a). It should be noted that many of the regions that were affected by genetic aberrations in TNBCs such as gain of 1q, 3q, 7q, 8q, and 10p and loss of 4p, 5q, 17p, and 8p, have also been found in tumours arising in *BRCA1* mutation carriers (Jonsson et al., 2005). These results highlight the similarities between sporadic TNBCs and tumours arising in *BRCA1* mutation carriers and provide yet another line of evidence to suggest that, as a group, TNBCs phenocopy familial *BRCA1* tumours (Turner *et al.*, 2004).

Similar to the HER2 amplicon, whose smallest regions of amplification encompasses 13 genes, of which only 7 are expressed at significantly higher levels when amplified (Marchio *et al.*, 2008; Orsetti *et al.*, 2004), our analysis demonstrated that only a few genes mapping to regions recurrently amplified in TN breast cancers were consistently overexpressed when amplified. Importantly, genes identified in this study as overexpressed when amplified may constitute potential therapeutic targets, including *FGFR2* (10q26), mitotic spindle

checkpoint protein *BUB3* (10q26), RAS oncogene family member *RAB20* (13q34), Notch family member *NOTCH3* (19p13) and the protein kinase C super family member *PKN1* (19p13).

Our data adds to the body of evidence linking aberrant FGF signaling to breast cancer pathogenesis. Interestingly, a SNP in *FGFR2* intron 2 is a common low risk predisposition gene for breast cancer, that predisposes selectively for ER positive breast cancer (Easton et al., 2007; Garcia-Closas et al., 2008). In contrast our data suggest that amplification of *FGFR2* is found in ER negative TNBCs. Most TNBCs exhibit high levels of genomic instability, which potentially reflects an underlying defect in the processes that maintain genome stability, and this defect could theoretically provide a mechanism through which some TNBCs acquire *FGFR2* amplfication, coupled with the survival advantage conferred by activated FGFR2 signalling. Activating mutations in *FGFR2* are found in endometrial cancer (Byron *et al.*, 2008), but are rare in breast cancer (Greenman *et al.*, 2007).

We confirmed that a mechanism underlying oncogene addiction in cell lines with *FGFR2* gene amplification is activation of PI3K-AKT signalling, and resulting inhibition of apoptosis. Signalling in cell lines harbouring *FGFR2* amplification appears to be ligand independent, with both cell lines activating AKT in an FGFR kinase dependent manner. Interestingly, in MFM223, AKT phosphorylation was predominantly under control of upstream FGFR signalling despite the presence of an activating *PI3KCA* kinase mutation (Figure 4), suggesting that in this cell line *PI3KCA* kinase domain mutation predominantly amplified upstream signal. Likewise SUM52 remained sensitive to FGFR inhibition, despite lacking PTEN expression (Supplementary Figure 5), concurring with previous results found in *FGFR2* mutant endometrial cancer cell lines (Byron *et al.*, 2008). We note that HCC1143 have been shown to harbour an *FGFR2* mutation (Greenman *et al.*, 2007). HCC1143 show no evidence of FGFR dependent signalling (Figure 4), nor dependence on FGFR for proliferation (Figure 3), suggesting the mutation identified (R203C) does not active the receptor.

Here, we have identified a higher number of recurrent amplifications in TN cancers than previously reported (Andre et al., 2009; Han et al., 2008), possibly due to the use of samples with >70% of tumour cells, which reduces the bias introduced by the contamination with diploid non-neoplastic cells (i.e. stromal cells and inflammatory infiltrate). In fact, 76.8% of the tumours analysed in this study harboured at least one focal (<10Mb) amplification. However, the majority of these amplifications were shown to occur at low frequency, confirming that at the genomic level TNBCs are genomically heterogeneous. To develop therapeutic strategies directed at drivers of the recurrent amplicons in TNBC will be challenging, requiring comprehensive molecular pathology analyses of primary tumours to select the appropriate targeted therapy for the individual tumours. A novel approach to clinical trial design would also be required if rare oncogenic targets are to be validated. An alternative approach to the therapy of TNBCs would be to identify shared signal transduction pathways that could be targeted for tumour treatment without targeting the oncogene directly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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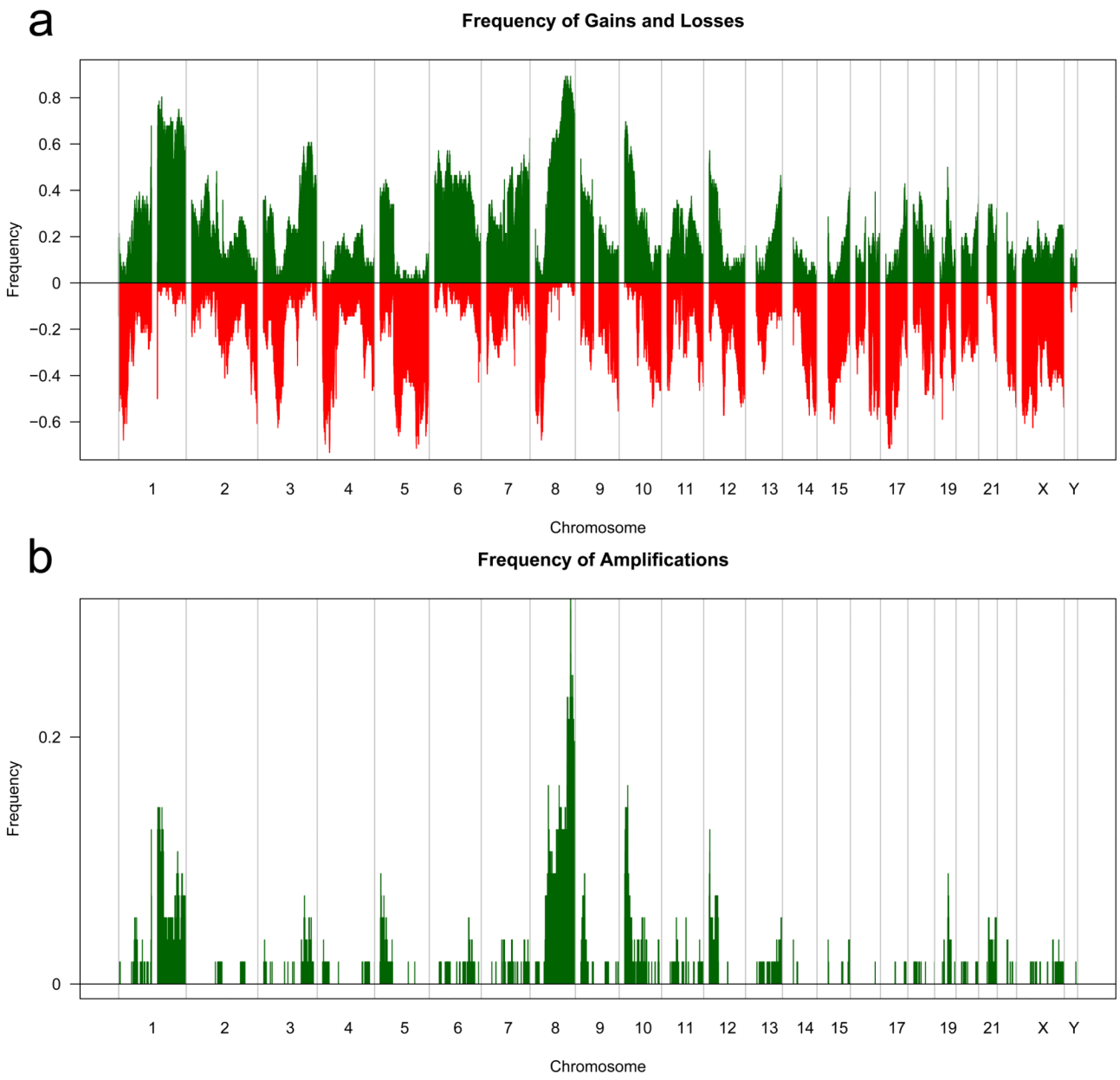

Figure 1.

Genomic alterations in triple negative breast cancers. a) Frequency of gains and losses in 56 TNBCs profiled with aCGH. The proportion of tumours in which each clone is gained (green bars) or lost (red bars) is plotted (Y axis) for each BAC clone according to genomic location (X axis). b) The proportion of tumours in which each clone is amplified (green bars) is plotted (Y axis) for each BAC clone according to genomic location (X axis). BAC clones were categorised as amplified if the ($\log_2$ ratio) cbs ratios were >0.45.

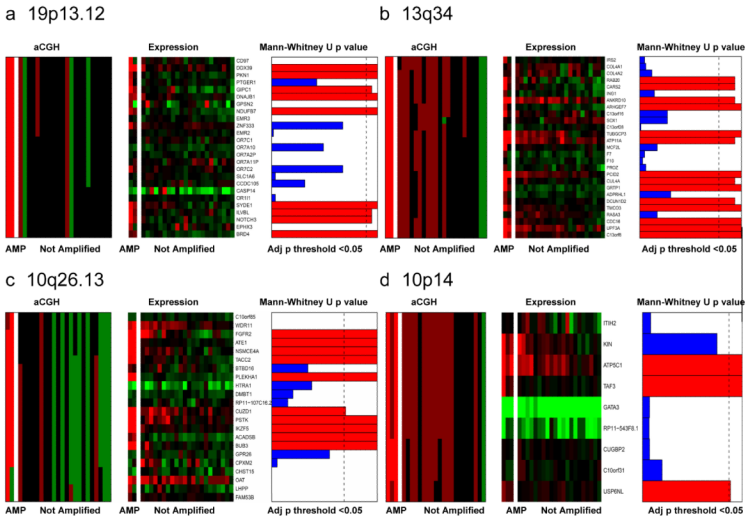

Figure 2.

Matched heatmaps of expression and aCGH within regions of recurrent amplification in 24 TNBCs. a) 19p13.12 amplicon; b) 13q34 amplicon; c) 10q26.13 amplicon; d) 10p14 amplicon. For each amplicon, genes within the amplified region are recovered and median aCGH values and states are assigned. Samples are separated into those harbouring an amplification within the region and those that do not. Expression and cbs values are depicted in two matching heatmaps (aCGH states on the left and expression values on the right) in which the genes are ordered according to their chromosomal position and the tumours ordered according to the sum of their aCGH values. In the correlation box, red bars indicate correlated genes whose expression significantly correlates with amplification (Mann-Whitney U test adjusted $p<0.05$), whereas blue bars indicate non significant genes (Mann-Whitney U test adjusted $p<0.05$). aCGH: green: copy number loss; black: no copy number change; dark red: copy number gain; bright red: gene amplification; gene expression: green: downregulation, red: upregulation.

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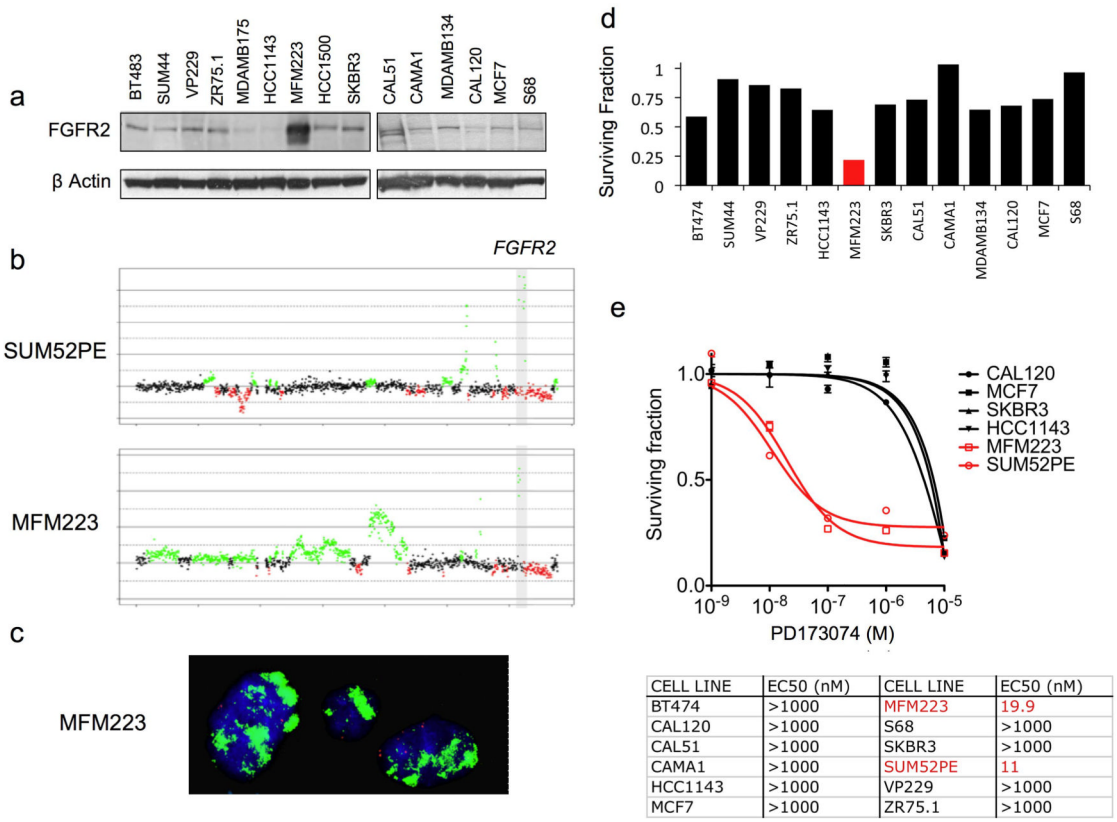

CELL LINE	EC50 (nM)	CELL LINE	EC50 (nM)
BT474	>1000	MFM223	19.9
CAL120	>1000	S68	>1000
CAL51	>1000	SKBR3	>1000
CAMA1	>1000	SUM52PE	11
HCC1143	>1000	VP229	>1000
MCF7	>1000	ZR75.1	>1000

Figure 3.

FGFR2 amplified cell lines are sensitive to *FGFR2* silencing and FGFR inhibition. a) Western blot of cell lines of 15 breast cancer cell lines, demonstrating over-expression of FGFR2 protein in MFM223. FGFR2 was frequently observed as a doublet, that reflects different glycosylation states of the extracellular domain. b) Chromosome 10 aCGH profiles of MFM223 and SUM52PE with a gray box indicating the smallest region of amplification of the *FGFR2* amplicon. c) FISH for chromosome 10 centromere (red) and *FGFR2* (green) on MFM223 cells. MFM223 demonstrate unquantifiable high numbers of *FGFR2* signals. The *FGFR2* probe was specific for the *FGFR2* locus (Supplementary Figure 2). d) Sensitivity of breast cancer cell lines to FGFR2 siRNA, demonstrating sensitivity of MFM223 (red) to FGFR2 silencing. Cell lines were transfected with FGFR2 siRNA, or siCON non-targeting control, and survival assessed at 5-7 days post transfection with Cell Titre-Glo® cell viability assay (Promega). Survival of FGFR2 siRNA transfected cells was expressed relative to that of siCON transfected. e) Graph: Selected cell lines were grown for 96 hrs in media supplemented with a range of concentrations of PD173074 pan FGFR tyrosine kinase inhibitor, and survival expressed relative to that of untreated cells. *FGFR2* amplified cell lines in Red. Error bars represent SEM. Table: IC50 of breast cancer cell lines to 96hrs treatment with PD173074.

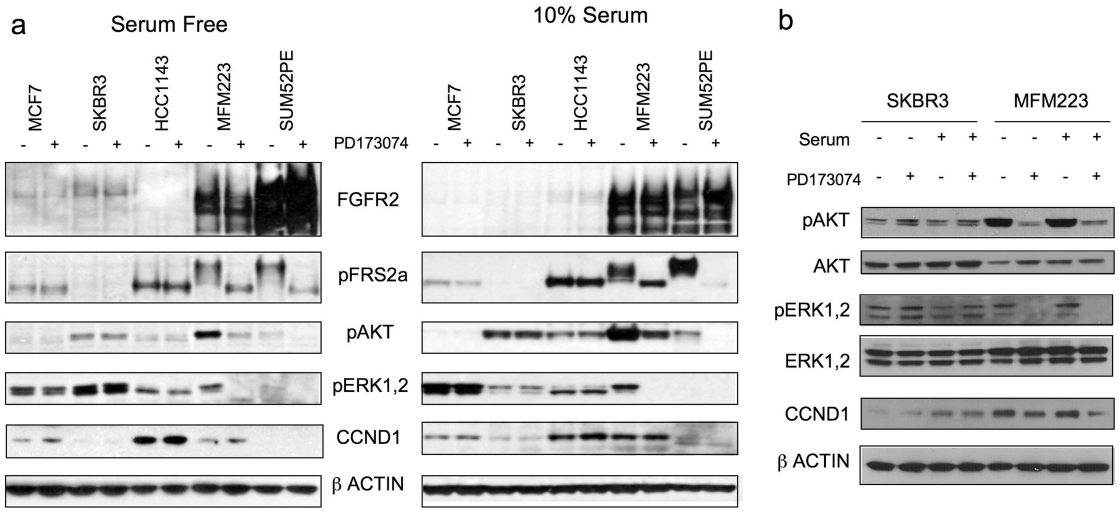

Figure 4.

a) Signalling downstream of FGFR2 in amplified cell lines. Indicated cell lines were grown either in 10% serum, or serum starved for 24 hrs, and lysates were made after 1hr exposure to 1μM PD173074 (+), or no exposure (−), as indicated. Lysates were subject to SDS-PAGE and western blotting with antibodies against FGFR2, phosphorylated FRS2-Tyr196, phosphorylated AKT1-Ser473, phosphorylated ERK1/2-Thr202/Tyr204, CCND1, and β-Actin. b) Side-by-side comparison of lysates from MFM223 grown in 10% serum or serum starved, with or without 1μM PD173074, with SKBR3 lysates for comparison.

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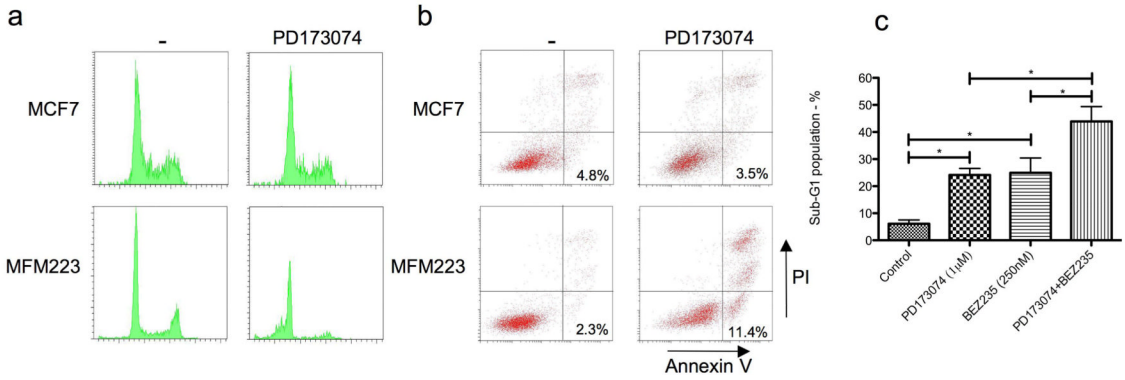

Figure 5.

FGFR inhibition induces apoptosis in MFM223 partly through loss of AKT signalling. a) PI cell cycle profiles in MCF7 and MFM223 treated with vehicle or 1μM PD173074 for 48 hrs. b) Annexin V / PI staining in MCF7 and MFM223 treated with vehicle or 1μM PD173074 for 48 hrs. Example plots from one experiment, with proportion of apoptotic Annexin V positive/PI negative cells from three independent experiments: MCF7 without and with PD173074 (4.8% vs 3.5% respectively, p=NS), MFM223 without and with PD173074 (2.3% vs 11.4% respectively, p<0.001 Student's T Test). c) Fraction of cells in subG1, as assessed by PI FACS, after 24 hours exposure to 1μM PD173074, BEZ235 250nM, or combination of both inhibitors. Displayed mean of three independent experiments. Error bars SEM and * P<0.01 (Student's T test).

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Table 1

Tyrosine kinase receptor canonical pathways significantly enriched for genes significantly upregulated when gained.

Ingenuity Canonical Pathways	p	Ratio	Genes
Angiopoietin Signaling	0.0030	0.0735	PTK2,PAK1,FOXO1,PIK3C3,BIRC5
HGF Signaling	0.0047	0.0594	PTK2,RAF1,PAK1,GAB1,PIK3C3,MAPK9
FAK Signaling	0.0102	0.0532	PTK2,RAF1,PAK1,PIK3C3,CAPN10
FGF Signaling	0.0380	0.0476	RAF1,GAB1,PIK3C3,MAPKAPK2
VEGF Signaling	0.0427	0.0449	PTK2,RAF1,FOXO1,PIK3C3
IGF-1 Signaling	0.0490	0.0426	PTK2,RAF1,FOXO1,PIK3C3

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Table 2

Genes that are significantly over-expressed when amplified.

Symbol	Description	Cytoband	Start (Mb)	End (Mb)	MWU Adjusted p value amplification
SIPA1L2	Signal-induced proliferation-associated 1-like protein 2	1q42.2	232533711	232697304	0.029
IMPA1	Inositol monophosphatase A1	8q21.13	82569151	82598589	0.024
ZFAND1	AN1-type zinc finger protein 1	8q21.13	82613958	82633530	0.024
SNX16	Sorting nexin-16	8q21.13	82711822	82754521	0.024
ATP5C1	ATP synthase subunit gamma, mitochondrial Precursor	10p14	7830093	7849755	0.031
TAF3	Transcription initiation factor TFIID subunit 3	10p14	7860501	8056714	0.031
USP6NL	USP6 N-terminal-like protein	10p14	11502509	11653753	0.047
FGFR2	Fibroblast growth factor receptor 2 Precursor	10q26.13	123237848	123357972	0.013
ATE1	Arginyl-tRNA--protein transferase 1	10q26.13	123502626	123687977	0.013
NSMCE4A	Non-SMC element 4 homolog A	10q26.13	123716611	123734710	0.013
TACC2	Transforming acidic coiled-coil-containing protein 2	10q26.13	123748709	124014059	0.013
PLEKHA1	Pleckstrin homology domain-containing family A member 1	10q26.13	124134220	124191867	0.013
CUZD1	CUB and zona pellucida-like domain-containing protein 1 Precursor	10q26.13	124591665	124610309	0.047
PSTK	L-seryl-tRNA(Sec) kinase	10q26.13	124739556	124749908	0.013
IKZF5	Zinc finger protein Pegasus	10q26.13	124751965	124768321	0.013
ACADSB	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial Precursor	10q26.13	124768429	124817806	0.013
BUB3	Budding uninhibited by benzimidazoles 3 homolog	10q26.13	124913760	124924886	0.013
RAB20	Ras-related protein Rab-20	13q34	111175419	111214080	0.027
CARS2	Cysteinyl-tRNA synthetase 2, mitochondrial	13q34	111293759	111358463	0.027
ANKRD10	Ankyrin repeat domain-containing protein 10	13q34	111530887	111567416	0.027
ARHGEF7	Rho guanine nucleotide exchange factor 7	13q34	111767624	111958078	0.021
TUBGCP3	Gamma-tubulin complex component 3	13q34	113139326	113242481	0.021
ATP11A	Probable phospholipid-transporting ATPase IH	13q34	113344643	113541482	0.027
PCID2	PCI domain-containing protein 2	13q34	113831891	113863029	0.027

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Symbol	Description	Cytoband	Start (Mb)	End (Mb)	MWU Adjusted p value amplification
CUL4A	Cullin-4A	13q34	113863086	113919399	0.021
GRTP1	Growth hormone-regulated TBC protein 1	13q34	113978506	114018463	0.021
DCUN1D2	DCN1-like protein 2	13q34	114110134	114145023	0.027
TMCO3	Transmembrane and coiled-coil domain-containing protein 3 Precursor	13q34	114145308	114204542	0.021
CDC16	Cell division cycle protein 16 homolog	13q34	115000362	115038198	0.021
UPF3A	Regulator of nonsense transcripts 3A	13q34	115047078	115071261	0.021
C13orf8	Zinc finger protein 828	13q34	115079988	115092796	0.021
DDX39	ATP-dependent RNA helicase	19p13.12	14519633	14530171	0.035
PKN1	Serine/threonine-protein kinase N1	19p13.12	14544166	14582678	0.035
GIPC1	PDZ domain-containing protein	19p13.12	14588572	14606944	0.042
DNAJB1	DnaJ homolog subfamily B member 1	19p13.12	14625582	14629201	0.035
NDUFB7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	19p13.12	14676892	14682886	0.035
SYDE1	Rho GTPase-activating protein	19p13.12	15218214	15225789	0.035
ILVBL	Acetolactate synthase-like protein	19p13.12	15225789	15236577	0.042
NOTCH3	Neurogenic locus notch homolog protein 3 Precursor	19p13.12	15270445	15311792	0.042
BRD4	Bromodomain-containing protein 4	19p13.12	15348301	15391262	0.035

MWU: Mann Whitney U test.