Journal of Cancer Research and Clinical Oncology (2019) 145:821–828
https://doi.org/10.1007/s00432-018-02833-8

ORIGINAL ARTICLE – CANCER RESEARCH

Differential molecular pathways expression in HER2 positive early breast cancer according to hormone receptor status

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Received: 26 November 2018 / Accepted: 21 December 2018 / Published online: 2 January 2019

Abstract

Purpose Hormone receptors (HR) status in HER2 + breast cancer (BC) is a recognized stratification factor with relevant clinical implication. According to HR expression, HER2 + BC show different clinical characteristics, treatment sensitivity and prognosis. The interaction between HR and HER2 pathways remains incompletely understood.

Methods Thirty-four HER2 + BC were included: 18 tumors with HER2+/HR + and 16 with HER2+/HR−. The expression of 770 genes and 13 molecular pathways were evaluated using Nanostring PanCancer Pathway panel performed on FFPE BC biopsies.

Results Gene expression analysis identified 127 genes with significantly different expression between the two cohorts. 83% of these genes were overexpressed in HER2+/HR− cohort. Globally, 23% of them belonged to PI3K pathway (41 genes), 15% to Trascriptional regulation (26 genes) and 12% to MAPK (22 genes). Regarding pathway expression, PI3K, MAPK and NOTCH were significantly differently expressed between the two groups ($p = 0.003$, $p = 0.0018$ and $p = 0.02$, respectively), all of them were overexpressed in HER2+/HR− tumors.

Conclusions According to HR status, HER2 + tumors express different pathways profiles: the overexpression of PI3K, MAPK and NOTCH pathways in HER2+/HR− group could justify different survival outcomes and treatment sensitivity. The identification of tumor driver pathways may be a useful instrument for individualized pathway-directed therapies. Further clinical implications are warranted.

Keywords HER2 positive · Trastuzumab · PI3K · MAPK · Molecular pathways

Background

Breast cancer (BC) with amplification and/or overexpression of Human Epidermal growth factor Receptor 2 (HER2+) oncogene are about 15% of the BC diagnosis. Poor prognostic clinical features and aggressive behavior characterize HER2 + tumors (Escriva-de-Romani et al. 2018). Several studies suggest that HER2 + BC subtype is biologically and clinically heterogeneous. The co-expression of hormone receptors (HR) may partially account for such heterogeneity. Published reports underline how HER2 + BCs have different clinical characteristics, treatment sensitivity and prognosis according to HR status (Blows et al. 2010; Garcia Fernandez et al. 2012; Parise et al. 2009). Results from neoadjuvant trials suggest that the HR status influences the anti-HER2 treatment sensitivity and induces chemo-resistance (Baselga et al. 2012; Gianni et al. 2010). Indeed, according to PAM50 signature, up to one-third of HER2 + BC might be intrinsically classified as luminal-like disease with high endocrine responsiveness (Gomez Pardo 2011; Cheang 2011). Actually, HR status in HER2 + tumors is considered a stratification factor with relevant clinical implications even if the

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interaction between HR and HER2 pathways remain incompletely understood.

In the recent years, researchers have made great efforts to find biomarkers that could characterize and differentiate BC subtypes. Multigene tests are a useful tool to better understand BC biology (Cancer Genome Atlas 2012). Based on gene expression data, it is possible to discover molecular pathway aberrations and consequently to identify the main pathways involved in tumorigenesis process (Omarini et al. 2018). This knowledge provides an opportunity to better understand the cancer behaviour and further define personalized treatment strategies in each patient.

The present study has been designed to investigate molecular pathways differentiating HER2 positive/HR positive (HER2+/HR+) BCs from HER2 positive/ HR negative (HER2+/HR−) ones. We have analysed the expression of 770 genes and 13 molecular pathways on formalin-fixed paraffin-embedded (FFPE) tissues from BC biopsy, using Nanostring PanCancer Pathway panel.

Materials and methods

Population

Thirty-four patients diagnosed with HER2 + BC at Modena Cancer Center and with available FFPE BC tissues from breast biopsy were considered. Tumors samples were collected before any systemic anticancer treatment. HER2 and HR status was assessed on the primary BC tissue using immunohistochemistry and/or in situ hybridization (ISH) according to international guidelines. HR− tumors have been defined as neither estrogen nor progesterone receptors expression detected (the cut of level was 0%). As of 34 patients included in the study, 18 and 16 patients were HER2+/HR+ (Group 1) and HER2+/HR− (Group 2), respectively.

The local Ethical Committee authorized this mono-institutional retrospective study; alive patients signed a written consent form.

RNA isolation and gene expression analysis

RNA was extracted from FFPE of BC core biopsies. The hematoxylin and eosin stained sections were reviewed by breast-pathologist and areas containing not less than 20 mm-square of invasive breast carcinoma were outlined on the slides. After removal of the not neoplastic breast tissue by manual microdissection, the RNA was extracted from two to four 10 µm sections. RNA extracted was purified using High Pure FFPE RNA Isolation Kit according to manufacturer's instructions. RNA concentration was measured by spectrophotometry using a Xpose Instrument (Trinean).

Gene expression on the nCounter platform (prep Station and Digital Analyzer—Nanostring technology, Seattle, USA) was assessed with the nCounter PanCancer Pathway panel. The panel analyses the expression of 770 genes (606 Pathway genes, 124 Driver genes and 40 Housekeeping genes) involved into 13 molecular pathways (Notch, Wnt, Hedgehog, TGFβ, MAPK, STAT, PI3K, RAS, Chromatin Modification, Transcriptional Regulation, DNA Damage Control, Cell Cycle and Apoptosis). The platform estimates the quantity of each mRNA transcript using a multiplexed hybridization system and digital readouts of fluorescent barcoded probes that are hybridized to each transcription. Row counts resulting from the analysis were normalized against reference genes, genes selected to have the least variance with the geNorm algorithm. Normalized data were analysed using NanoString's nSolver version 3.0 software with the Advanced Analysis application tool based on Pathifier algorithm (Drier et al. 2013). Using this algorithm, genes that were significantly up- or down-regulated between patients group were identified (p value ≤ 0.05). Genes expression was graphically represented by a heat-map. The same algorithm was used to calculate the molecular pathways deregulation score for each tumor sample based on gene expression data. A boxplots for each pathways was designed using pathways deregulation score.

Results

A total of 34 BC patients were included in the study: 18 patients with HER2+/HR + and 16 with HER2+/HR− disease. Considering the clinical characterizes of the patients enrolled, all of them received the same adjuvant/neoadjuvant treatments (anthracycline–taxane-based chemotherapy plus trastuzumab). None patients relapsed at the time of the analysis.

Whole genome expression analysis comparing HER2+/HR + versus HER2+/HR− identified 127 genes with significantly different expression between the two cohorts (p value < 0.05, Fig. 1; Table 1). Only 27% of these genes (34 genes) were overexpressed in both groups while 10% (13 genes) were down regulated in both cohorts. Most of these genes are overexpressed in HER2+/HR− compared to HER2+/HR + patients, in particular 105 genes were significantly overexpressed in HR− subgroup. The majority of them encoded growth factors, DNA repair factors or transcriptional factors. In HER2+/HR + cohort, 84 genes were down regulated; more than half of them were involved in four molecular pathways (17% in Trascriptional regulation pathway, 15% in STAT pathway and 13% in PI3K and MAPK, respectively). Nine genes overexpressed in HER2+/HR + cancers were down regulated in the HER2+/

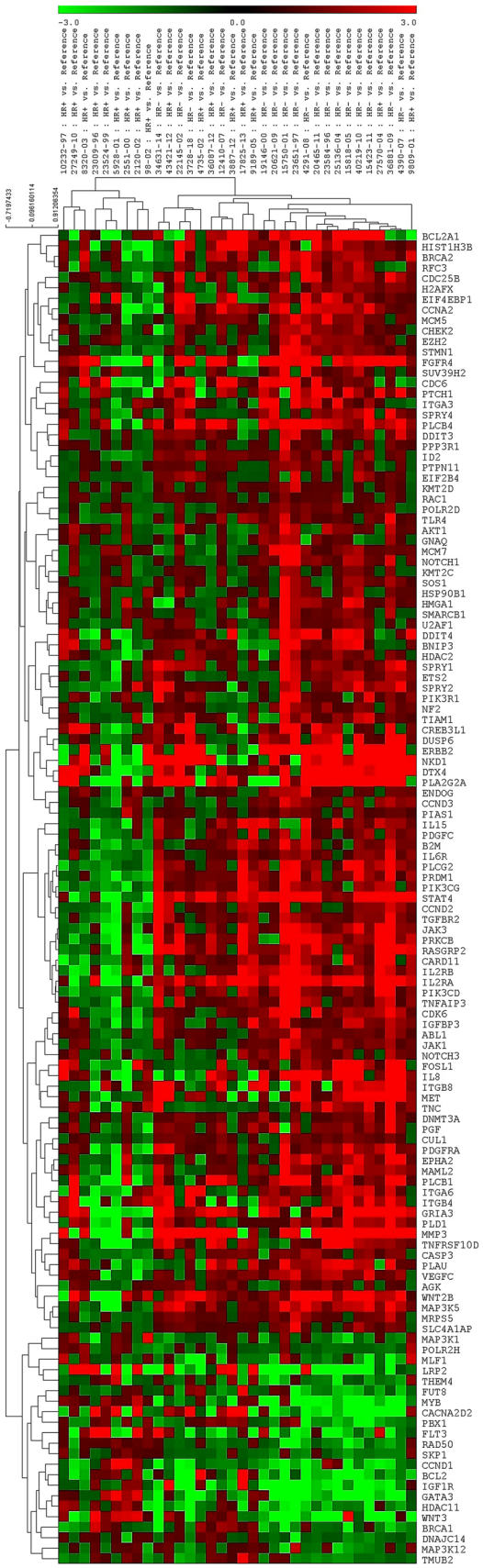

Fig. 1 Hierarchical clustering based on RNA expression levels of 127 genes out of 770 genes analysed by PanCancer Pathways panel. Rows, genes; columns: samples. Expression level of each gene in a single sample was related to its median level across all samples and is depicted according to a colour scale show above. Red and green represented the expression levels above and below the median, respectively

HR− (MAP3K1, MAP3K5, MYB, PBX1, RAD50, HDAC11, DNAJC14, MAP3K1, TMUN2).

Overall, among the genes differently expressed in the two cohorts, 23% belonged to PI3K (41 genes), 15% to Trascriptional regulation (26 genes) and 12% to MAPK (22 genes) pathways (Fig. 2). No gene involved in Hedgehog pathway was detected. Regarding pathway expression, PI3K, MAPK and NOTCH were significantly and differently expressed between the two groups ($p = 0.03$, $p = 0.001$ and $p = 0.03$, respectively) (Fig. 3; Table 2). In particular, all these three pathways were overexpressed in HER2+/HR− cohort. Considering PI3K pathway, 82% of the genes (34 genes) were strongly overexpressed in HER2+/HR− subgroup compared to 34% (14 genes) in HER2+/HR + patients. All but one of the 22 genes involved in MAPK were overexpressed in HER2+/HR− group. Only four differently expressed genes belong to NOCTH pathway, all in HER2+/HR− cohort (Fig. 4). Even if not statistically significant, APC, TGFβ and Cell cycle were the only three pathways up regulated in HER2+/HR + compare to HER2+/HR− group.

Discussion

HER2 + BC is not a heterogeneous disease; tumor biology is not fully reflected by the main immunohistochemical biomarkers routinely used in clinic. HR co-expression may partially account for such heterogeneity. Approximately, half of the cancers with HER2 + disease are also HR+ (Vici et al. 2015). Considering treatment sensitivity, it is commonly believed that anti-HER2 agents are effective in HER2 + disease irrespective of HR status. Data from subgroup analysis of clinical trials underlined how HER2+/HR + disease showed less benefit from endocrine therapy than women with HER2-/HR + tumors (Montemurro et al. 2013). Likewise, data from the efficacy of HER2-targeted agents in early stage BC have shown how HER2+/HR− disease were more sensitive to treatment compare to HER2+/HR + subgroup (Baselga et al. 2012; Gianni et al. 2012; Untch et al. 2012). Preclinical studies corroborate the role played by the functional crosstalk between HER2 and HR signalling in chemo and endocrine resistance: HR expression seems to correlate to HER2 blocking agents resistance such as HER2 positivity played a negative role in hormonal treatments efficacy (Shou et al. 2004; Witters et al. 2002; Liu et al. 2009; Xia et al.

Table 1 Genes with significantly different expression between HER2+/HR + and HER2+/HR− breast cancer

Pathway	Genes					
STAT	AKT1	IL15	IL2RA	IL2RB	IL6R	IL8
	MET	PTPN11	RAC1	SPRY1	SPRY2	SPRY4
	CCND1	JAK3	PIAS1	JAK1	STAT4	
MAPK	AKT1	CREB3L1	CARD11	CASP3	CDC25B	DDIT3
	FGFR4	MAP3K1	MAP3K5	PPP3R1	PRDM1	PRKCB
	STMN1	TGFBR2	AGK	MAP3K12	TMUB2	SOS1
	RASGRP2	DTX4	RAC1	DUSP6		
RAS	ABL1	ETS2	EZH2	FGFR4	PDGFC	PDGFRA
	PLD1	PTPN11	PLA2G2A	SOS1	TIAM1	VEGFC
	PLA2G2A	RASGRP2	PGF	IGF1R		
Cell Cycle	ABL1	CCNA2	CCND2	CCND3	CDC25B	CDC6
	CUL1	HDAC2	MCM5	MCM7	SKP1	CCND1
	CDK6					
Apoptosis	AKT1	ENDOG	EPHA2	PPP3R1	SMARCB1	TNFAIP3
	TNFRSF10D	BCL2				
NOTCH	HDAC2	MAML2	NOTCH1	NOTCH3		
Trascriptional Regulation	BCL2A1	CCND2	CCND3	DDIT3	DUSP6	FLT3
	GNAQ	GRIA3	HDAC2	MLF1	HMGA1	IGFBP3
	MMP3	MYB	PBX1	PLAU	TGFBR2	EIF2B4
	IGF1R	GATA3	MRPS5	HIST1H3B	SLC4A1AP	FOSL1
	MET	FUT8				
PI3K	B2M	CDK6	CREB3L1	DDIT4	DNMT3A	FGFR4
	HSP90B1	ID2	IL2RA	IL2RB	IL6R	IL8
	ITGB4	ITGB8	JAK1	JAK3	MAP3K1	MAP3K5
	PDGFRA	PGF	PIK3CD	PIK3CG	PIK3R1	THEM4
	TLR4	TNC	VEGFC	BCL2	DNAJC14	IGF1R
	BRCA1	EIF4EBP1	ITGA6	PDGFC	CCND1	MAP3K12
	ERBB2	ITGA3	MET	SOS1	RAC1	
Cromatin Modification	BNIP3	H2AFX	HDAC2	SUV39H2	HDAC11	
DNA Damage Control	BRCA2	POLR2D	POLR2H	RAD50	RFC3	BRCA1
APC	CUL1	KMT2C	KMT2D	LRP2	NF2	NKD1
	PLCG2	PPP3R1	PTCH1	SKP1	U2AF1	WNT2B
	PLCB4	PLCB1	WNT3			
TGFβ	CUL1	SKP1	TGFBR2			

Genes are classified according to pathway involvement

2006). This lead to the question about whether HR status defines two distinct subtypes of HER2 positive BC.

On these premises, we focused our study on the molecular profile of HER2 positive BC according to hormonal receptors status. Using PanCancer Pathway panel, we analysed the expression of 770 genes and 13 molecular pathways in HER2+/HR + and HER2+/HR− cohorts of HER2 positive BC. We looked for discriminate different pathways between the two groups, to identify different cellular mechanisms that could be the drivers of tumorigenesis. No data regarding gene mutations or rearrangements, gene copy number variation and gene methylation have been detected by our molecular analysis. In our opinion, considering the expression of the main molecular pathways instead of the single genes expression/mutations, offers a clearer overview of the anarchic architecture of BC. Nowadays, it is well known how the detection of a single gene mutation cannot explain the extreme complexity of cancer biology that it is the result of several mutated genes and epigenetic phenomena. For these reasons, these cellular modifications could be more represented by the pathways expression data.

Our analysis identified three molecular pathways with significantly different expression in the two cohorts of HER+/HR + and HER2+/HR− BC: PI3K, MAPK and NOTCH. All of them were overexpressed in HER2+/HR− subgroup. These pathways might explain the known different treatment sensitivity and survival outcome of HER2+/HR + and HER-/HR− disease.

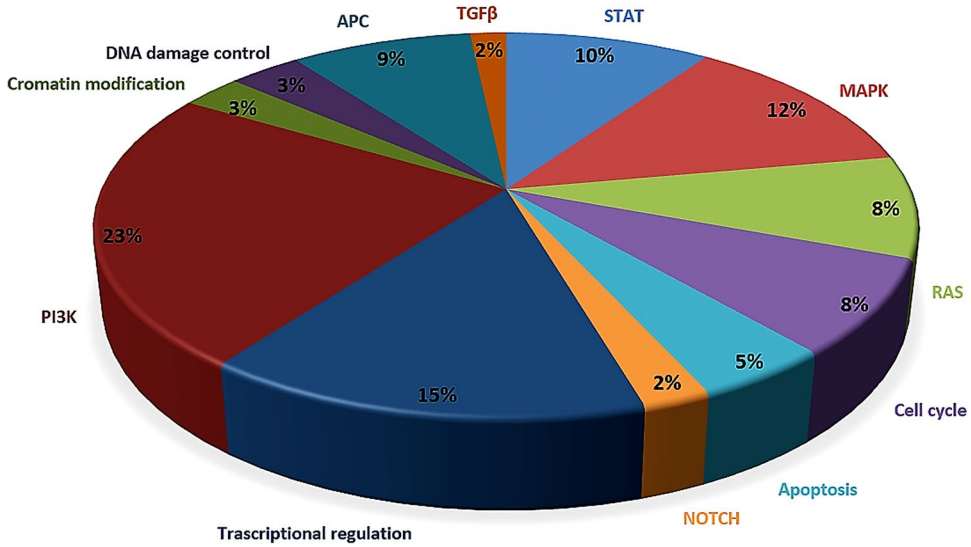

Fig. 2 Distribution of the 127 genes differently expressed between the HER2+/HR + and HER2+/HR− groups within the molecular pathways

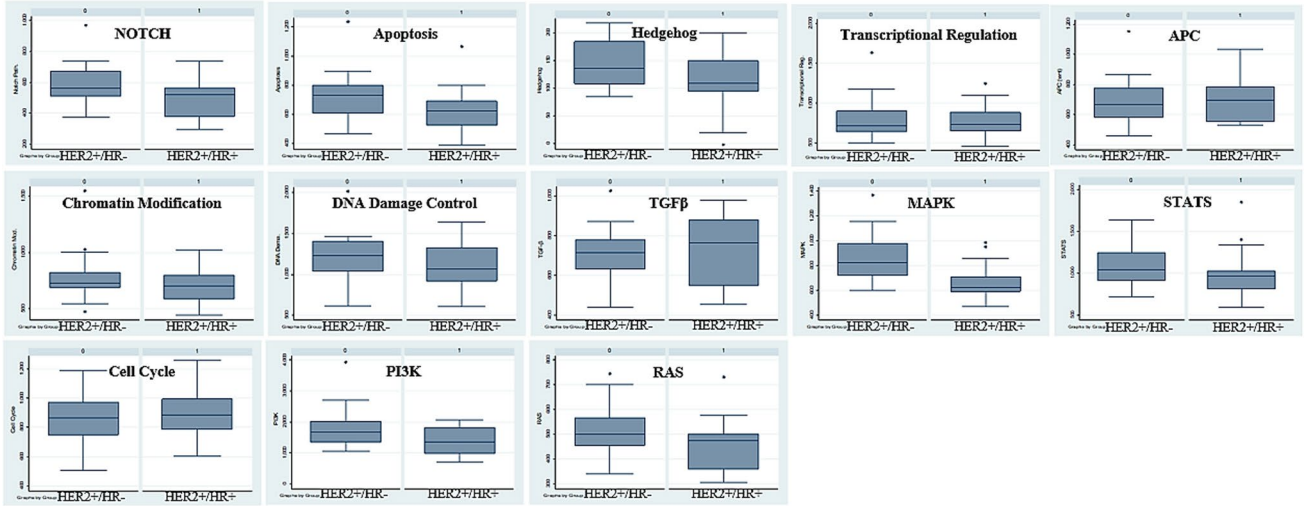

Fig. 3 Boxplots of pathways deregulation scores. The distribution of Pathifier deregulation scores of each pathways is plotted for HER2+/HR− and HER2+/HR+. The top and the bottom of the box delineate the upper and lower quartile, while the thick line within each box represents the median. Whiskers extend capture all data within two standard deviations of the means

Considering PI3K pathway, the overexpression of PI3KCA is commonly observed in BC and correlated with drug-resistance. Several studies have shown that the expression and the activation of PI3K are frequently observed in trastuzumab-refractory cancers, justifying their more aggressive behaviour (Nahta 2012). Clinical data from Bolero-3 and Bolero-1 trials have shown that the addiction of everolimus to anti-HER2 agents significantly improve progression free survival in the subgroup of HER2+/HR− patients (Hurvitz et al. 2015; Andre et al. 2014). The advantage due to the double inhibition of PI3K and HER2 pathways suggests that in HER2+/HR− group, PI3K acts as an escape pathway when HER2 is inhibited. So, our evidence that HER2+/HR− tumors presented an overexpression of PI3K pathway compare to HER2+/HR + ones could support the evidences from Bolero trials.

Regarding MAPK pathway, it is a signalling cascade hyper-activated in a large number of cancers. Cross talk between MAPK and HER2 cascade is through the activation of ERBB-family receptors. Biologically ERBB receptor activates membrane bound RAS, allowing RAS to bind multiple effector proteins such as RAF, MEK1 and ERK (Kirouac et al. 2016). Preclinical data suggest that the dual inhibition of PI3K and MAPK cascade can results in synergic effects on cells proliferation and apoptosis in HER2 + BC cells, suggesting a potential role of MAPK signalling in in the growth and survival of HER2 + BC (Serra et al. 2011; Yarden and Pines 2012). Moreover, interesting studies have

Table 2 Pathway deregulation score in HER2+/HR + patients and HER2+/HR− patients

Pathways	HER2+/HR+ Media ± SD (CI95%)	HER2+/HR− Media ± SD (CI95%)	*p* value
NOTCH	491 ± 122 (431–552)	596 ± 140 (521–671)	**0.03**
APC	703 ± 152 (627–779)	694 ± 167 (605–783)	0.86
Hedgehog	115 ± 52 (89–141)	143 ± 44 (119–167)	0.10
Chromatin Mod	695 ± 153 (619–771)	797 ± 244 (667–927)	0.15
Transcriptional Reg	777 ± 209 (672–880)	815 ± 277 (667–963)	0.65
DNA damage control	1100 ± 255 (973–1227)	1227 ± 323 (1054–1398)	0.21
TBFβ	720 ± 171 (635–805)	710 ± 136 (638–783)	0.84
MAPK	667 ± 141 (597–737)	875 ± 211 (763–987)	**0.001**
STAT	980 ± 301 (830–1130)	1096 ± 281 (947–1246)	0.25
PI3K	1361 ± 446 (1139–1583)	1810 ± 720 (1427–2194)	**0.03**
RAS	461 ± 110 (406–516)	521 ± 103 (466–575)	0.11
Cell Cycle	890 ± 158 (811–968)	860 ± 188 (760–960)	0.61
Apoptosis	626 ± 159 (547–705)	735 ± 176 (641–829)	0.06

Bold values indicate the statistically significant *p* value

SD Standard deviation; *CI95%* Confidence Interval

found that PI3KCA mutated HER2 + tumors escape PI3KCA dependence by activating MAPK/MEK signalling pathway (Yarden and Pines 2012). A strong rationale and preclinical results have established the groundwork for the clinical development of dual PI3K and MEK inhibitor therapy to treat solid malignancies. However, early-phase clinical trials presented to date have only shown modest activity of the combination. It is possible that efficiency of dual targeting could be increased by a selection of patients. To the best of our knowledge, our findings are the first data regarding the overexpression of MAPK pathway in HER2+/HR− BC compare to HER+/HR + ones, suggesting that MAPK could increase its activity when hormonal signalling is ineffective. This data identify HER2 + HR− subgroup as an optimal candidate for PI3K and MEK inhibitor therapies.

The interaction between NOTCH and HER2 pathways has been investigated in preclinical studies. It is well known that NOTCH controls the differentiation of breast epithelial cells during normal development as well as NOTCH pathway plays a major role in BC progression through the overexpression of NOTCH receptors and ligands that determine neoangiogenesis (Witters et al. 2002). Furthermore, the overexpression of NOTCH cascade seems to correlate to treatment sensitivity. In vitro studies showed that BC cells resistant to anti-HER2 therapy had both NOTCH-1 expression and NOTCH transcriptional target genes increased, while the inhibition of NOTCH-1 enhanced trastuzumab sensitivity. These findings led to the conclusion that HER2 inhibition increased NOCTH-1 expression in a compensatory manner to promote survival and resistance of HER2 + cancer cells (Xia 2006). At the same time, NOTCH-3 seems to be involved in the early steps of tumorigenesis. Targeted inhibition of NOTCH-3 reduces early tumor progression in DCIS-HER2 + lesions (Pradeep et al. 2012). In our experience, HER2+/HR− group demonstrated high expression of NOTCH-1 and NOTCH-3, supporting an increased cross talk between NOTCH and HER2 pathways.

Overall, our findings suggest that the overexpression of PI3K, MAPK and NOTCH pathways in HER2+/HR− cohort could be a possible reason for the different biological behaviour of HER2 + tumors group. All these three signalling cascades are involved in bidirectional crosstalk within HER2 family and could play a critical role in treatment sensitivity and tumorigenesis process. Understanding the molecular pathways by which oncogenes drive cells growth, and how dependence on such pathways varies between tumors could be highly valuable for the design of anti-cancer treatment strategies.

Conclusion

In the era of precision medicine, continue to regard all the HER2 + BCs as the same disease with the same treatment options is outdated. HR expression stratifies HER2 + disease in two different BCs subtypes. Biologically these tumors have different driver pathways that lead the tumorigenesis process. To the best of our knowledge, this is the first study that performed a molecular analysis, comparing the expression of 770 genes and 13 pathways, in HER2+/HR + versus HER2+/HR− disease. The overexpression of PI3K, MAPK and NOTCH pathways in HER2+/HR− group could justify for different survival outcomes and treatment sensitivity, with respect to HER2+/HR + cohort. The identification of tumor driver pathways may be a useful instrument for individualized pathway-directed therapies and may be a major

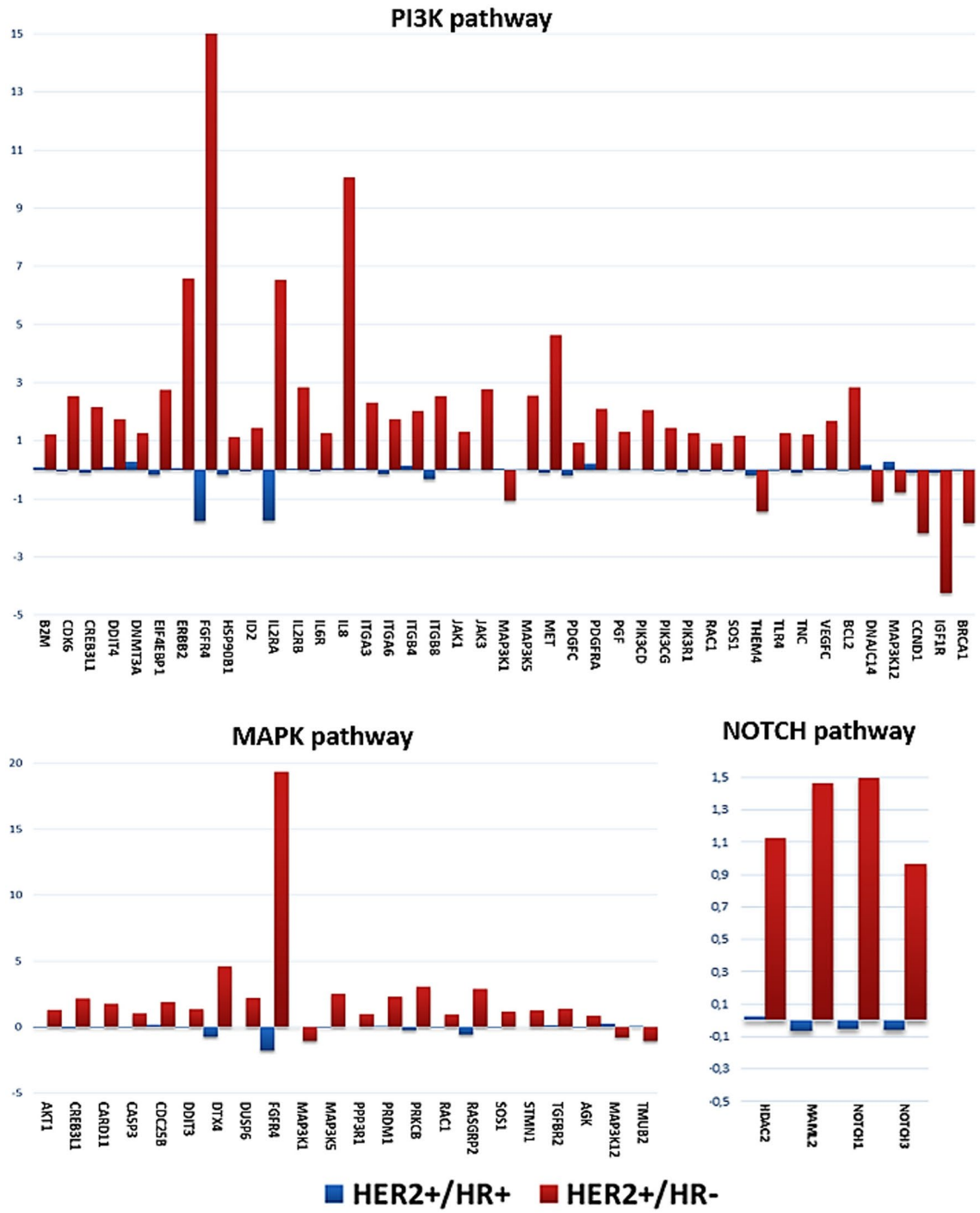

Fig. 4 Genes differently expressed between the HER2+/HR + and HER2+/HR− involved PI3K, MAPK and NOTCH pathway

step in defining optimal treatments for every patient. Considering the small sample size and the retrospective nature of the study our results are purely exploratory and need to be validate in larger studies.

Funding This study was funded by Progetto Ricerca Finalizzata 2009 (RF 2009-1472600).

Compliance with ethical standards

Conflict of interest No potential conflicts of interest were declared.

Ethics approval and consent to participate The Ethical Committee of Azienda Ospedaliero Universitaria Policlinico di Modena approved this study (protocol number: CE 267/15). All patients signed a written, informed consent.

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