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MOLECULAR AND MICROSPECTROSCOPIC INTEGRAL PROFILES OF BREAST CARCINOMAS AND THEIR RESISTANCE TO NEOADJUVANT TREATMENT

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1. Abstract

Neoadjuvant treatment is a strategy increasingly used in breast carcinomas, especially in the HER2+ and triple negative subtypes (TNBC) achieving a complete pathological response rate around 30-50% of cases according to different studies. This has led to a great advance in the prognosis of this disease and is associated with a greater survival in these subtypes. However, a percentage of patients do not respond well to neoadjuvant treatment, and the mechanisms underlying resistance to this type of therapy remain largely unclear. Among them, we are beginning to know certain genetic and phenotypic alterations that are still scarcely defined.

In this project we intended to analyze a series of patients with primary infiltrating mammary carcinomas that included retrospective and prospective cases of Her2 + and triple negative phenotypes treated with neoadjuvant chemotherapy and that consisted of good (complete pathological response, cPR) and poor responders. The existence of alterations in several processes and pathways was analyzed by means of a broad mRNA expression panel (nCounter ®BrestCancer 360 panel) with a multigenic platform (nCounter, Nanostring Technologies Inc.). This transcriptional characterization was carried out in pretreatment biopsy samples and, in cases without cPR, in the residual tumor. In some cases, paired distant metastases were also considered in order to allow typing throughout several phases of tumor progression. The type of pre- and post-treatment inflammatory response was also analyzed, as well as the expression of the PD-L1 marker in tumor and inflammatory cells. Molecular findings were validated in in vitro and in vivo models by using naive and resistant breast carcinoma cell lines. Raman microspectroscopy was tested as a novel tool for the typification of breast samples and its use in clinical settings was analyzed.

2. Results

In the present study we identified 214 candidate cases: 117 HER2+ (60 of them achieved complete pathological response) and 97 TNBC (49 with complete pathological response). The transcriptomic profile was characterized by using the nCounter Breast Cancer 360 Panel (Nanostring Technologies) that contains 776 human genes across 23 key breast cancer pathways. In total we finally analyzed 124 human samples: 71

HER2+, 31 of them with cPR, and 40 with partial response, 17 being post-treatment biopsy; and 53 TNBC, 20 of them with cPR and 33 with partial or poor response, 17 being post-treatment biopsies and 1 distant metastasis. We also characterized the transcriptomic profile for 14 cell lines: eight HER2+ and six TNBC parental and resistant to different treatments. We performed differential gene expression analysis based on both genes and biological signatures. We identified several significant downregulated genes (*AGTR1, LAMA3, SCUBE2, SLC39A6, TBC1D9, IFT140*) and one upregulated gene (*MARCO*) in the HER2+ responders' pre-treated biopsies compared to the poor or non-responders' pretreated samples.

When we performed the comparison for the TN pre-NAT cohort (pCR 20; NO pCR=14), no significantly different expression has been observed so far in any gene between responders and non-responders, although more cases are being added.
We also identified in the HER2+ cohort, significant overexpressed genes (*FOS, NR4A1, NR4A3, IL6, PDK4, OGN, FHL1, GADD45B, FGF2, JUN, FLNC, LPL* and *SPRY*) in the post-neoadjuvant group compared to the pretreatment biopsies that did not respond to treatment. When we compared the TNBC cohort, we obtained similar results: overexpression of *FOS, NR4A3, NR4A1, IL6, HBB, PDK4, JUN* and *GADD45*.

We analyzed the possible association between immunohistochemical assays and clinical and pathological parameters. Our results seem to indicate that tumors with high-TILs present a significantly higher histological grade and are associated with a better response to treatment.

The comparative analysis of these tumor groups and samples in terms of the expression of biological signatures is currently being carried out.

On the other hand, we developed HER2+ resistant to trastuzumab and lapatinib cell lines (MDA-MB-453, SK-BR-3 and BT-474) and two lung metastatic cell lines derived from a lung metastasis model in immunosuppressed mice (MDA-MB-453). We also developed three TNBC resistant to paclitaxel or adriamycin cell lines: MDA-MB-468, classified as basal A BC cell line, and BT-549 and MDA-MB-231 as claudin-low tumors (Basal B).

The resistant cell lines displayed a multidrug resistant phenotype associated to the overexpression of the ABC-type transporters such as ABCB1 (multidrug resistance 1 MDR1 / P glycoprotein Pgp) and ABCG2 (breast cancer resistance protein BCRP). These resistant cell lines developed a more oxidative metabolic strategy, a reduction of the proliferative signaling and resistance to autophagia and apoptosis, in agreement with the acquired resistant phenotype. We characterized the transcriptomic profile of these resistant cell lines and their parentals. The gene expression of these cells seemed to be very different compared to the human tumor samples. This could be related to the fact that cell lines are composed of homogeneous group of cells (tumor cells), whereas human biopsies contain not only tumor cells but also tumor microenvironment including stromal and inflammatory cells that can play also an important role in cancer development.

In addition, by in vitro analysis we have confirmed some members of the NR4A family as putative common mediators of chemotherapy resistance in TNBC and on the metastatic potential of HER2+ subtypes. We have also unveiled the relevance of the HBB gene in the resistant phenotypes, especially related to HER2-targeted therapies and HER2+ metastatic potential. In agreement with our results, overexpression of *NR4A1* and *HBB* has been previously related with a poor survival and bad prognosis in different cancers, including breast cancer, however, they had never related to treatment response prediction. With our results, we propose NR4A1 and HBB as potential biomarker predictors to neoadjuvant treatment resistance in HER2+ and TNBC patients.

By implementing Raman spectroscopy and multivariate analysis, we have been able to classify with excellent accuracy wild-type non-resistant cells from cells of the same line that developed resistant to neoadjuvant treatments. For HER2+ cells, non-resistant MDA-MB-453 and BT474 cells were distinguished from the cells resistant to lapatinib and trastuzumab treatments, and detected significant changes in their phospholipid, DNA and cytochrome C abundances. Similarly, for triple negative cells, non-resistant MDA-MB-231 and BT549 cells were distinguished from the cells resistant to paclitaxel and adriamycin treatments and detected significant changes in their lipid unsaturation levels. The chemical information provided was extracted non-destructively and will help to unravel treatment resistance mechanisms that are not completely understood yet. Also, by implementing the same technique, a protocol to compare Raman

measurements of tissue biopsy cuts and cylindrical core needle biopsies (tru-cuts) with their later histopathological analysis, has been established. Its potential to identify different tissue sections according to their protein, lipidic, collagen and carotene content has been shown, which allows the differentiation of healthy tissue from cancerous tissue, further validated with later histopathological analysis. With further work, this technique could help doctors in real-time on the assessment of biopsy quality and cancer diagnosis.

3. Relevance with possible future implications

Several oncogenes and tumor suppressor genes (such as *AGTR1*, *LAMA3*, *SLC39A6*, *SCUBE2*) were identified as significantly and differentially regulated when tumor samples which responded were compared with those not responding to neoadjuvant treatment. Once validated in upcoming experiments which go beyond this project (i.e. with immunohistochemistry techniques and clinical follow-up) they have the potential to serve as tumor biomarkers predictive of response in HER2+ and TNBC tumors. Experimental confirmation both in vitro and in vivo will be also completed with clinical prospective analysis of the expression of these markers in pretreatment samples of breast cancer patients. If these biomarkers prove clinically predictive capacity for current neoadjuvant drugs (i.e. anti-Her2, taxanes), a routine use in daily management of these two types of breast cancer could be proposed, which could affect around 1,000 women in Catalonia per year and improve their survival by better adjusting their treatment.

On the other hand, the finding of genes differentially expressed in the same tumor prior to and after the treatment (such as *NR4A1*, *NR4A3* and *HBB*) helps to better understand the metabolic changes affecting cancer cells after neoadjuvant treatment thus helping to develop new therapeutical approaches and drugs for breast cancer treatment.

Other biomarkers such as PD-L1 and TILs seem to be associated with response prediction. Due to their easy implementation, we believe that we can now use them in the management of these patients. Indeed, we now routinely include TILs information in the diagnostic reports of invasive carcinomas.

In addition, our project has proved the feasibility of analyzing both cell lines and fresh breast tumor samples by means of RAMAN and obtaining important tissue section composition (protein, lipidic, collagen and carotene content) and metabolic information related to resistance to drugs in a non-destructive manner. This provides a novel diagnostic approach to the management of breast cancer also applicable to other types of tumors. Moreover, the viable use on needle biopsies indicates that RAMAN microspectroscopy has the potential for implementation in routine clinical practice once our results are confirmed in larger series and more manageable devices are designed and deployed at hospital premises.

4. Scientific bibliography generated

Articles

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Marro M. *Label-free molecular monitoring, screening, and imaging of human cells and tissues by Raman spectroscopy*. BIST Conference: Precision Medicine: Putting discoveries to work. Flash Talk

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