Allegato/Annex B

RESEARCH PROJECT

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Project title	Dissecting the transcriptome of CD44 in triple negative breast cancer
PhD	Oncologia e chirurgia sperimentali
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1 - Abstract

Breast cancer (BC) encompasses various histotypes differing in genetic and clinical features and can be categorized into in situ carcinomas and invasive carcinomas. The histological classification of BC based on the evaluation of morphological features such as growth patterns and cytological features, allows to distinguish special types of both in situ and infiltrating carcinomas.
Among the infiltrating BCs, non-specific invasive ductal carcinoma represents the most frequent main histotype (60-75%), while 20-25% include lobular, tubular, papillary, and mucinous carcinomas [1].
In 2000, Perou and Sorlie introduced a molecular classification demonstrating for the first time a high grade of variability in gene expression within BCs. The proposed classification identified molecular classes based on the expression of estrogen (ER), progesterone (PR) and Her-2 Neu: luminal-like, basal-like, normal-like, and HER-2 positive [2]. Subsequently, luminal class was further subclassified into Luminal A and Luminal B, forming a fifth class of BCs.
Luminal A (LumA) is the most common subtype of BCs and it is characterized by estrogen (ER) and/or progesterone (PR) receptor expression with a favorable outcome. Compared to other subtypes, LumA hardly presents a local recurrence [3] with ki-67 index lower than in other subtypes [4].
Luminal B (LumB) subtype is characterized by a lower expression of estrogen/progesterone receptors than LumA and presents a higher histologic grade with an increased expression of proliferative markers compared to LumA [5].
The Her-2 class comprises 25% of BCs and reflects ErbB2 amplification and HER-2 neu overexpression. It has been related to a higher aggressive biological behavior compared to luminal class, with unfavorable outcome.
Normal-like BC is a molecular class which presents analogies to luminal A disease, indeed it is hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive), HER2 negative and shows low levels of Ki-67. Even if this group have a good prognosis, it is slightly worse than luminal A subtype's one.
Finally, the Triple Negative BC (TNBC) subtype represents approximately 10–15% of all BCs. It is defined by the lack of expression of estrogen, progesterone and HER2 neu receptors. TNBC is a group of tumors with an aggressive biological behavior reflecting on its tendency to metastasize with a higher likelihood of brain and lung involvement and a poorer outcome compared to other BC classes.

Gene expression profiling allowed to identify different subtypes within TBNC according the molecular characteristics, such as basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptors [6].
Burtsein et al suggested another classification for TNBC including luminal androgen receptor, mesenchymal, basal-like immune-suppressed and basal-like immune-activated [7].
In the current era, the molecular classification provided important biological insights into tumoral heterogeneity and emphasized the biological complexity within BCs, stratifying patients in cancer subtyping which is important for selecting patients who can benefit from specific therapies and for identifying new potential drug targets.
The status of these markers determine the responsivity of targeted therapies (i.e., tamoxifen or aromatase inhibitors for ER^+/PR^+ patients and trastuzumab or lapatinib for HER2/neu patients) [8].
Furthermore, the College of American Pathologists recommend the use of gene array profiling or immunohistochemical staining for ER, PR and HER2-neu to be applied in the clinical practice [9].
CD44 is a transmembrane glycoprotein expressed on embryonic stem cells and on stromal cells of the connective tissue and bone marrow, as well as on hematopoietic cells [10].
It plays an important role in the cross-talk between cell and the extracellular matrix (ECM).
The biological function of the CD44 receptor is to operate like a sensor involved in the recognition of specific ECM ligands that activate cellular transduction signal pathways.
Among its ligands, notable elements are osteopontin, collagens and matrix metalloproteinases [11]
Hyaluronic acid (HA), expressed by mesenchymal elements and neoplastic cells, which represent the major interactors [12].
The HA-CD44 binding turns on cell signaling pathways leading cell proliferation, increased cell survival, and enhanced cellular motility, adhesion, migration, and invasion [13].
The CD44 has a complex transcriptional regulation; indeed, many isoforms of CD44 have been described. CD44 standard isoform is encoded by ten constant exons, while CD44 variant isoforms (CD44v) are generated by alternative splicing and result constituted by the ten constant exons and any combination of the remaining nine variant exons [14].
Some of these isoforms are transcribed specifically in a tissue, while other ones are encoded in different tissues with variable levels of expression [15]. Furthermore, in silico analyses allowed to predict potential CD44 transcripts.
The region most frequently involved in the splicing codifies for the ligand binding site [11], leading a different specificity and affinity of each isoform variant for the CD44 ligands. [15].
Furthermore, CD44 is also overexpressed in several cancer stem cell types and frequently shows alternative spliced variants which seem to play a role in tumorigenesis and neoplastic progression.
CD44s and its isoforms play a role in the promoting tumorigenesis and may be considered as a molecular target for new therapeutic approaches and a potential prognostic biomarker [16].
Neoplastic elements that undergo an epithelial to mesenchymal transition (EMT) display increased CD44 expression [17] and acquire an aggressive behavior with a higher grade of invasiveness and resistance to chemotherapy [18].
Several studies correlate CD44v with specify tumor subtypes, particularly the V6 isoform (CD44v6). It is mainly expressed in epithelia, even if high levels are detected in the majority of squamous cell carcinomas and a variable percentage of adenocarcinomas with diverse differentiation phenotypes [19]. For example, CD44v6 seems to be related to disease progression in gastric and colon cancer, determining an unfavorable outcome [20].
Recent studies highlighted a key role of CD44 in breast cancer progression. The binding CD44-ECM ligands promotes the cleavages in either extracellular and cytoplasmic sites of the protein, with the formation of the two extracellular (ECD) and intracellular (ICD) domain fragments.

Thorne et al. showed that ICD translocates into the nucleus and turns on the transcription of genes strictly involved in cell survival and metastasis [21]. Nevertheless, the underlying mechanisms result unclear and need further elucidations.
The aim of this project is to dissect the CD44 transcriptome in cell TNBC cell lines characterized by a different malignant biological phenotype and in primary TNBC human tissue samples, in order to identify new potential transcriptional variants and shed light on the diagnostic, therapeutic and prognostic significance of CD44 constellation.

2 - Project

In situ immunolocalization analyses, PCR assays, sequencing technologies including Sanger sequencing and next generation sequencing (NGS) and in vivo experiments will be performed to detect new potential CD44 isoforms, using two prototypical TNBC cell lines, namely the MDA-MB-468 and MDA-MB-231.
In 1977 Cailleau isolated the MDA-MB-468 cell line from a female patient presenting advanced breast carcinoma associated to metastasis [22]. The MDA-MB-468 line presents the morphological features of basal-like phenotype, including a low grade of differentiation, a high histological grade with marked cellular pleomorphism, a high nuclear-cytoplasmic ratio, vesicular chromatin, prominent nucleoli and frequent apoptotic figures. It is also characterized by a strong EGFR and cytokeratin 5/6 expression with a high Ki-67 index. Despite these biological characteristics, this cell line shows a good therapeutic responsiveness.
The MDA-MB-231 was obtained from a pleural effusion of a female patient with a metastatic breast cancer [23]. It is recognized as the claudin-low molecular subtype, exhibiting down-regulation of claudin-3 and claudinin-4. The MDA-MB-231 also presents low expression of the Ki-67 proliferation marker and enrichment for markers associated with the epithelial-to-mesenchymal transition. Moreover, the MDA-MB-231 immunophenotype relates to mammary cancer stem cells, with a high expression of CD44 and a low expression of CD24 [24].
Since in xenograft models, MDA-MB-231 cells are able to metastasize to the bones, brain and lungs, they are well established as a tool to identify genes and pathways that are potential mediators of metastasis to specific sites [25-26-27-28]. The MDA-MB-231cell line shows a high degradation potential through proteolytic activity on the ECM, for this reason being considered as a highly aggressive malignant neoplasm. Moreover, it is characterized by an intermediate response to chemotherapy.

The proposed study will begin with immunophenotypic characterization of both the MDA-MB-468 and MDA-MB-231 by immunohistochemical analyses. In details, different antibodies recognizing CD44 specific and variable sequences will be employed.
Successively, from RNA extracted from the two cell lines, PCR assays will allow to enrich the CD44 transcriptome with the generation of the *library* comprising all the possible transcripts of the CD44 gene. Each library will be sequenced through an NGS platform. Potential new transcripts will be validated by PCR-based approaches and through Sanger sequencing and an in depth comparative analysis of the transcriptomes of the two cell lines will be performed.
The experimental plan of the proposed research project also included functional experiments using *ad hoc*-designed siRNA targeted against specific candidate CD44 isoforms and several readouts of the silencing on cell lines biology will be pursued. These will include analyses of the cell viability and proliferative capacity in vitro, sensitivity to chemotherapy agents adopted in TNBC clinical practice and ability to orthotopically seed and progress in in vivo experiments.
The finding of new CD44 isoforms, discovery of novel interactomes, and/or identification of biological correlates of specific CD44 isoform silencing through in vitro and in vivo models

experiments will be validated in a large cohort of primary human TNBCs (93 cases) selected from the archives of the IRCCS Fondazione Istituto Nazionale Tumori di Milano, which are available at the hosting Laboratory.
The MDA-MB-468 and MDA-MB-231 cells, all the required platforms, facilities and consumables as well as bioinformatics support for the accomplishment of the proposed research will be provided by the Tumor Immunology Laboratory of the University of Palermo, which is funded by Italian Foundation for Cancer Research, Italian Ministry of Research and Education, and Cancer Research UK grants. The experimental activity of the proponent will be constantly mentored by the Head of the Hosting Laboratory, Professor Claudio Tripodo, MD.

3 - References

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