1 **Exploring the metastatic role of the inhibitor of apoptosis BIRC6 in Breast**

2 **Cancer**

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13

14   Abstract

15   Breast cancer is the most common cancer as well as the first cause of death by cancer in

16   women worldwide. BIRC6 (baculoviral IAP repeat-containing protein 6) is a member of the

17   inhibitors of apoptosis protein family thought to play an important role in the progression or

18   chemoresistance of many cancers. The aim of the present work was to investigate the role of

19   apoptosis inhibitor BIRC6 in breast cancer, focusing particularly on its involvement in the

20   metastatic cascade.

21   We analyzed BIRC6 mRNA expression levels and Copy Number Variations (CNV) in three

22   breast cancer databases from The Cancer Genome Atlas (TCGA) comparing clinical and

23   molecular attributes. Genomic analysis was performed using CBioportal platform while

24   transcriptomic studies (mRNA expression levels, correlation heatmaps, survival plots and

25   Gene Ontology) were performed with USC Xena and R. Statistical significance was set at p-

26   values less than 0.05.

27   Our analyses showed that there was a differential expression of BIRC6 in cancer samples

28   when compared to normal samples. CNV that involve amplification and gain of BIRC6 gene

29   were correlated with negative hormone receptor tumors, higher prognostic indexes, younger

30   age at diagnosis and both chemotherapy and radiotherapy administration. Transcriptomic and

31   gene-ontology analyses showed that, in conditions of high BIRC6 mRNA levels, there are

32   differential expression patterns in apoptotic, proliferation, and metastatic pathways.

33   In summary, our *in silico* analyses suggest that BIRC6 exhibits an antiapoptotic, pro-

34   proliferative and an apparent pro-metastatic role and could be a relevant molecular target for

35   treatment of Breast Cancer tumors.

1. Introduction

Breast cancer (BC) is one of the most prevalent cancers in the general population, and it's the leading cause of death for female cancer patients. This pathology presents heterogeneity in the biological behavior of tumors and a great clinical variability (1). The main cause of breast cancer-related death is the development of metastasis, which accounts for 90% of deaths. The recurrence of this disease originates in local processes on secondary organs and is associated with a poor prognosis (2). The main characteristic that differentiates a benign tumor from a malignant tumor is the invasiveness of the malignant cells. This ability to invade surrounding tissues is fundamental for metastasis. Said process comprises several steps, all of which are controlled by different cellular and environmental signals (3). Understanding metastasis is of central importance when searching for antitumor therapies since identification of potential molecular targets for treatment requires discerning the key factors involved in it. Apoptosis is a highly regulated process and its failure can result in many pathological conditions including tumor development. In mammals, programmed cell death is usually regulated by the IAP family of proteins (named after their main function, IAP: "inhibitors of apoptosis proteins") (4). IAP, which were originally isolated and characterized from baculoviral genomes, contain highly conserved protein-protein interaction motifs called baculoviral IAP repeats (BIR). Through these BIR motifs, IAP are able to associate with different caspases and prevent their activity, thereby inhibiting apoptosis. Many IAPs also contain a RING domain at their C-terminal end with E3 ubiquitin ligase activity that allows control of protein levels by ubiquitination and degradation via proteasome (5). This family of proteins plays a central role in the control of survival and programmed cell death by regulating determining factors in both the caspase activation pathway as well as the NF-κB pathway (5).

The role of IAPs in tumor progression and metastasis has been reported for several tumor types (6–9). Recently, a PanCancer transcriptomic analysis showed a key role for IAPs in tumor physiology (10).

62   BIRC6 (Baculoviral IAP repeat-containing 6, also known as Apollon or BRUCE in mice) is a

63   IAP of approximately 530 kDa that contains a BIR domain at its N-terminal region and an

64   ubiquitin ligase domain at its C-terminal (UBC). Different research studies have described that

65   BIRC6 plays a dual role as an anti-apoptotic IAP and as a chimeric E2/E3 ubiquitin ligase.

66   BIRC6 is capable of catalyzing ubiquitination of different target proteins such as

67   SMAC/DIABLO and Caspase-9, among others (11–13). BIRC6 not only inhibits the pro-

68   apoptotic protein SMAC, but also binds to procaspase-9 and prevents its cleavage (13,14).

69   Likewise, through its BIR domain it can bind and inhibit active caspases, including caspases

70   3, 6, 7 and 9 (13–16).

71   In addition to its function as an inhibitory protein of apoptosis, BIRC6 plays both an important

72   role in cell proliferation and as a regulator of cytokinesis (17). BIRC6 is associated with the

73   membrane and is located in the Golgi compartments and in the vesicular system (16). BIRC6

74   also participates in other cellular processes such as autophagy, in which it regulates

75   autophagosome-lysosome fusion (18,19).

76   Different groups have demonstrated that IAP are overexpressed in several types of tumor

77   cells, and it has been inferred that they could be related to tumorigenesis, treatment

78   resistance, worse prognosis and oncogenesis (5,7,20–30). In particular, BIRC6

79   overexpression has been found in tumor tissues of gastric carcinomas (31), colorectal cancer

80   (21), breast cancer (22), and lung cancer (24) among others. These findings postulate IAP,

81   and particularly BIRC6, as a potential therapeutic target against different cancers, especially

82   those that most frequently develop chemoresistance. Such is the case of BC, for which BIRC6

83   has not yet been completely validated as a therapeutic target. Our aim was to further evaluate

84   whether BIRC6 may play a role in BC and metastasis using bioinformatic tools.

85   **2. Materials and methods**

86   **2.1 Breast Cancer and normal tissue samples**

87   We used different public datasets containing clinical, genomic and transcriptomic information

88   from patient samples. We also used two different platforms to analyze the data.

4

89   For these analyses, the TCGA and GTEx databases and the UCSCXena platform were

90   employed. Samples corresponding to mammary tissues were filtered and the expression of

91   BIRC6 and other genes was evaluated in conditions of normal tissue and tissue of primary

92   tumors.

93   **2.2 CNV and clinical attributes**

94   For these analyses, the BC database METABRIC (32,33) and the cBioPortal platform were

95   used. The BIRC6 gene was used as a query and the correlation of different copy number

96   variations (CNV) with the expression of BIRC6 and several clinical attributes of interest was

97   assessed.

98   **2.3 Survival plots**

99   The UCSCXena (34) platform and the TCGA Pan-Cancer (35–38) database were used to

100   analyze the patients survival. To this end, samples corresponding to BC were filtered and the

101   expression of BIRC6 were evaluated. In the analysis, the samples were divided into two

102   groups: high (≥11.1) and low expression of BIRC6 (<11.1), and survival rates were plotted.

103   **2.4 Transcriptomic analyses**

104   The UCSCXena (34) and cBioPortal (39,40) platforms and the TCGA GTEx, TCGA Pan-

105   Cancer (35–38) and Molecular Taxonomy of BC International Consortium (BC, METABRIC)

106   databases were used to evaluate the expression of BIRC6 (32,33). TCGA GTEx was selected

107   because it is the only one that includes transcriptomic data from normal tissue from healthy

108   volunteers and tissue from primary tumors obtained from patients with BC. The TCGA Pan-

109   Cancer (PANCAN) and BC (METABRIC (32,33)) databases were selected on the basis of the

110   number of samples included in the datasets, and the different parameters that could be

111   evaluated. In the case of TCGA Pan-Cancer and TCGA-Target-GTEx, normalized RNAseqV2

112   data was employed using RSEM quantification (41). In the case of BC (METABRIC), the

113   mRNA expression data in z-scores relative to all samples (log microarray) carried out on the

114   Illumina HT-12 v3 platform (Illumina_Human_WG-v3) (32) was used.

115   For different pathway correlation analyses, the TCGA Pan-Cancer database (PANCAN) and

116   the UCSCXena and cBioPortal platforms were used. Samples corresponding to BC were

117   filtered and the expression of BIRC6 and different molecules involved in the metastatic

118   cascade pathways were evaluated. In the analysis, the samples were divided into two groups:

119   high expression of BIRC6 (≥11.1) and low expression of BIRC6 (<11.1). In addition, the

120   analysis was performed for different types of BC: hormone receptor positive and hormone

121   receptor negative. The cut-off points to consider HR positive were the following: ESR1 ≥ 10

122   and PGR ≥7 for RSEM normalized expression.

123   **2.5 Gene Ontology and pathway analysis**

124   Publicly available data corresponding to the TCGA-BC dataset was used to perform differential

125   gene expression analysis and Gene Ontology. BC harmonized data (hg38) in HTseq- Counts

126   (raw counts) format was downloaded from the TCGA database (https://portal.gdc.cancer.gov/)

127   using the GDCdownload function of the TCGABiolinks package (2.18.0) (42–44) in R (45).

128   The dataset contained raw, fully sequenced transcriptome data from 1,088 primary tumor

129   samples from BC patients.

130   To perform the differential expression analysis of genes, the samples were separated into two

131   groups: those that presented BIRC6 expression values greater than the median and those

132   that presented BIRC6 expression values lower than the median. All samples were normalized

133   and filtered using R / Bioconductor's TCGABiolinks package following the standard pipeline.

134   They were preprocessed using the TCGAanalyze_Preprocessing function and a correlation

135   cutoff of 0.6, then normalized with TCGAanalyze_Normalization using the GC content method,

136   and finally filtered using TCGAanalyze_Filtering by quantile as recommended.

137   For the enrichment analysis, the TCGAanalyze_EAcomplete function was applied using the

138   DEGs (Differential Expressed Gene) with a log(FC)>0 for overexpressed DEGs or log(FC)<0

139   for less expressed DEGs, in order to obtain the 3 ontologies of those genes, respectively (GO:

140   biological process, GO: cellular component, and GO: molecular function) and the pathways in

141   which they were involved. These results were plotted using the TCGAvisualize_EAbarplot

142   function, showing the 35 biological processes with the lowest FDR.

143   **2.6 Statistical analysis**

144   *Expression of BIRC6 in samples of healthy volunteers and samples of mammary tumor tissue*:

145   n = 1275 (n = 1099 primary tumors; n = 176 normal breast tissue); t-Test. *Expression of BIRC6*

146   *vs. copy number*: n = 2173 (n = 232 Deletions; n = 1818 Diploids; n = 111 Gains; n = 12

147   Amplifications); multiple ANOVA followed by TukeyHSD. *BIRC6 CNV vs. presence / absence*

148   *of receptors*: n = 2140 was used to evaluate the estrogen receptor (n = 1617 ER +; n = 523

149   ER-), n = 1980 to evaluate the progesterone receptor (n = 1040 PR +; n = 940 PR -) and n =

150   1980 to evaluate the HER2 receptor (n = 247 HER2 +; n = 1733 HER2 -); $\chi 2$ test grouping

151   CNV that implied an increase in the number of copies (gains and amplifications) and those

152   that did not imply it (deletions and diploids). *BIRC6 CNV vs. age at diagnosis and the*

153   *Nottingham Prognostic Index*: n = 2173 (n = 232 Deletions; n = 1818 Diploids; n = 111 Gains;

154   n = 12 Amplifications); multiple ANOVA followed by TukeyHSD. *BIRC6 CNV vs. Neoplastic*

155   *Histological Grade*: n = 2072 (n = 174 Grade 1; n = 851 Grade 2; n = 1047 Grade 3); $\chi 2$ test

156   grouping CNV that implied an increase in the number of copies of the gene (gains and

157   amplifications) and those that did not imply it (deletions and diploids). *BIRC6 CNV vs.*

158   *treatment with chemotherapy or radiotherapy*: n = 1980 (n = 1173 Treated; n = 807 Not

159   Treated) in the case of radiotherapy and n = 1980 (n = 411 Treated; n = 1569 Untreated) in

160   the case of chemotherapy; $\chi 2$ test by grouping those CNV that implied an increase in the

161   number of copies (gains and amplifications) and those that did not imply it (deletions and

162   diploids). *BIRC6 expression vs. proteins of different pathways*: n = 1211 (n = 697 High

163   Expression of BIRC6; n = 514 Low Expression of BIRC6); t-test. *Correlation analysis*:

164   Pearson's correlation coefficient. Statistical significance was considered when p-value did not

165   exceed 0.05 for all studies.

166   **3. Results**

167   **3.1 Patient cohort**

168   For clinical, genomic and transcriptomic analyses three different databases were employed.

169   Patient attributes of each database are summarized in Table 1. The numbers in each cell

170   indicate the number of patients with the corresponding attribute.

Clinical attribute		Database		
		METABRIC	TCGA, PanCancer	TCGA-BRCA 2021
Number of patients (BC)		2509	1084	1098
Age	≤ 50	567	294	297
	> 50	1926	756	766
Histological Grade	1	214	Not available	Not available
	2	976	Not available	Not available
	3	1198	Not available	Not available
ER Status	Positive	1825	Not available	Not available
	Negative	644	Not available	Not available
PR Status	Positive	1040	Not available	Not available
	Negative	940	Not available	Not available
HER2 Status	Positive	247	Not available	Not available
	Negative	1733	Not available	Not available
Hormone Therapy	Yes	1216	Not available	Not available
	No	764	Not available	Not available
Chemotherapy	Yes	412	Not available	1097
	No	1568	Not available	1097
Radiotherapy	Yes	1173	549	1097
	No	807	434	1097

171   **Table 1. Patient cohort.** Table 1 shows principal clinical attributes corresponding to each of three

172   databases used in this work. The number in each cell indicates the quantity of patients with clinical

173   attributes specified in column one.

174   **3.2 BIRC6 is differentially expressed in tumor samples**

175   In order to evaluate the role of BIRC6 in human BC samples, we proceeded to study

176   transcriptomic databases.

177   The expression of BIRC6 was compared in samples from primary tumors of BC patients and

178   normal tissue samples from healthy volunteers. The result is shown in Fig 1A. We observed a

179   statistically significant increase in the expression of BIRC6 in primary tumor samples in

180   comparison to normal tissue from healthy volunteers. Cancer patients had a median z-score

181   of 0.113, whilst normal tissue has a median z-score of -0.372.
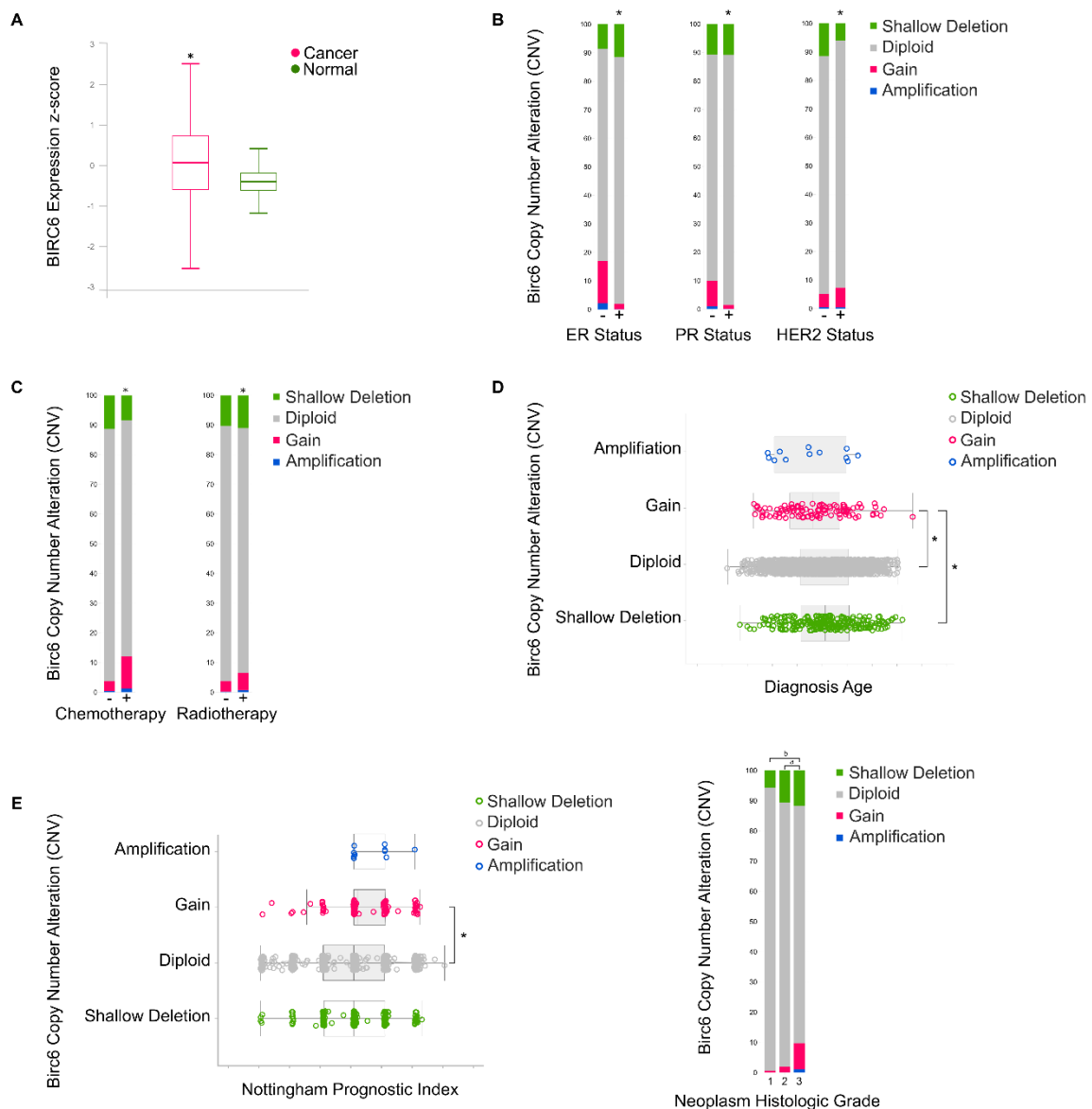
182

183   **Fig 1. BIRC6 expression and copy number variation (CNV).** (A) Differential expression of BIRC6

184   between tumor and normal tissue. (B) BIRC6 CNV abundance vs. different clinical attributes: ER, PR

185   and HER2 status, χ2 *p<0.05; (C) chemotherapy and radiotherapy, χ2 *p<0.05; (D) diagnosis age,

186   multiple ANOVA followed by TukeyHSD *p<0.05; (E) Notingham prognostic index, multiple ANOVA

187   followed by TukeyHSD *p<0.05 and neoplasm histologic grade, χ2 *p<0.05.

188   **3.3 Increased copy number of the BIRC6 gene correlates with higher cellular**

189   **dedifferentiation and worse prognosis**

190   To characterize whether the alteration of BIRC6 has an impact on the different clinical

191   attributes of patients we first evaluated if BIRC6 gene copy number variation (CNV) implied

192   any change of its expression level. The CNV data was divided into four groups: amplifications,

193   gains, diploids and deletions. A direct relationship was found between copy number and

194   mRNA expression. Samples with gene amplifications showed the highest expression levels

195   whereas genomic deletions were correlated with samples exhibiting the lowest expression

196   levels (S1 Fig).

197   Following these results, we decided to evaluate the relationship between CNV and the

198   presence or absence of estrogen (ER), progesterone (PR) and epidermal growth hormone 2

199   (HER2) receptors. The absence of these receptors in BC is associated with a much more

200   aggressive phenotype and an inability to use hormonal therapies to stop its development (Fig

201   1B). We observed a higher proportion of amplifications and gains of CNV in ER (-) and PR (-)

202   samples than in ER (+) and PR (+) samples, respectively.

203   We evaluated BIRC6 CNV distribution according to chemotherapy and radiotherapy

204   treatments. Fig 1C shows that in samples from patients who received either of the two

205   therapies, there was a greater amplification and gain percentage compared to those who did

206   not.

207   After evaluation of the average age at which patients with the different CNVs were diagnosed,

208   it became apparent that patients with higher CNVs tended to be diagnosed at a younger age

209   than those who maintained diploidy or had deletions in BIRC6 gene (Fig 1D). We assessed

210 the influence of CNV on two parameters that reflect prognosis: Nottingham Index and the

211 Neoplastic Histological Grade. It was observed that patients with amplifications had a higher

212 Nottingham Index than the rest of the conditions, thus implying worse prognosis (Fig 1E).

213 Furthermore, we found a higher proportion of patients with amplifications and gains of the

214 BIRC6 gene in patient samples with Histological Grade 3 (Fig 1E).

3.4 BIRC6 expression and survival

216 Breast cancer patient survival was evaluated using data of 1084 samples from TCGA

217 PanCancer Atlas. Patients were divided into two groups, high and low BIRC6 expression, and

218 survival was plotted for both groups. Survival time was significantly lower for patients with

219 higher BIRC6 expression levels (Fig 2A).
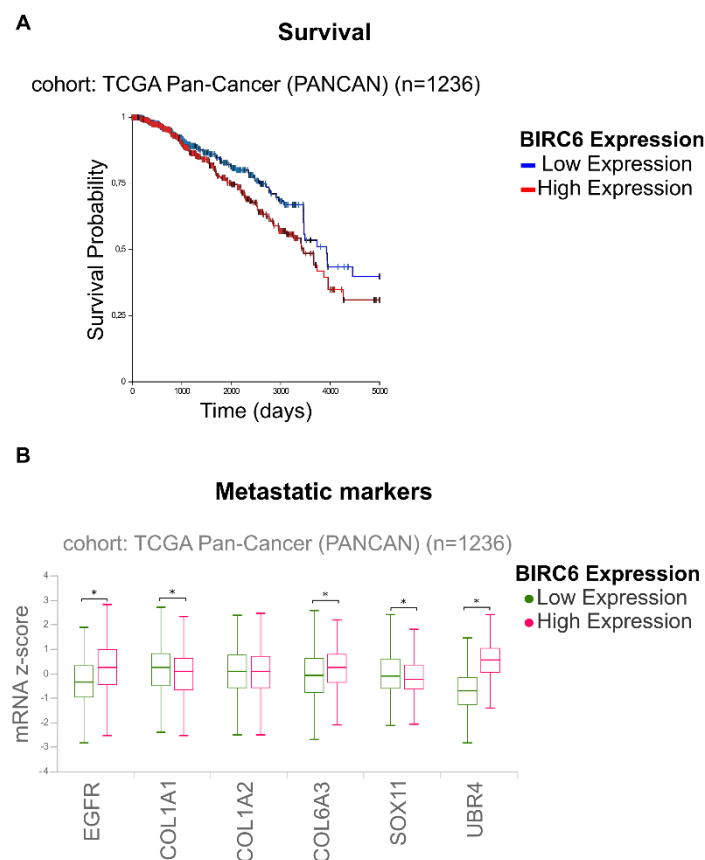
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221 **Fig 2. Survival and metastatic markers.** (A) Survival plot. BC samples were filtered from TCGA Pan-

222 Cancer and the expression of BIRC6 was evaluated. In the analysis, the samples were divided into two

223 groups: high expression of BIRC6 and low expression of BIRC6 and survival were plotted for each

11

224   group. T-test * p-value<0.05. (B) Metastatic markers expression. Boxplot of the expression (z-score) of

225   common metastatic markers in condition of high and low BIRC6 expression. T-test * p-value<0.01.

3.5 BIRC6 expression and pathways

227   Differential expression of six general metastasis markers (EGFR, COL1A1, COL1A2,

228   COL6A3, SOX11 and UBR4) was determined in patients with low and high expression of

229   BIRC6. EGFR, COL6A3 and UBR4 showed a significant increase in their expression levels in

230   samples with high BIRC6 expression (Fig 2B). Furthermore, we observed a significant

231   increase in LDHA and a significant decrease in PDH mRNA levels in samples with high

232   expression of BIRC6 in accordance with the expected metabolic switch for tumor cells (S2 Fig

233   and S1 Table). For a more systematic approach we conducted a Differential Expression

234   Analysis (DEA) followed by a gene ontology study. Differential genes obtained in this analysis

235   were divided in two output groups: overexpressed (Fig 3) and less expressed (S3 Fig) genes,

236   both under conditions of high BIRC6 expression levels. Fig 3 summarizes biological

237   processes, cellular components, molecular function and pathways in which overexpressed

238   genes are involved. We found that genes involved in gene expression (nucleosome and

239   chromatin assembly and organization, protein-DNA complexes and DNA packaging) were

240   overexpressed in the analyzed conditions as well as those involved in actin and myosin

241   cytoskeleton signaling, epithelial adherence signaling and tight junction signaling.

DEA genes Low Expression vs. BIRC6 High Expression
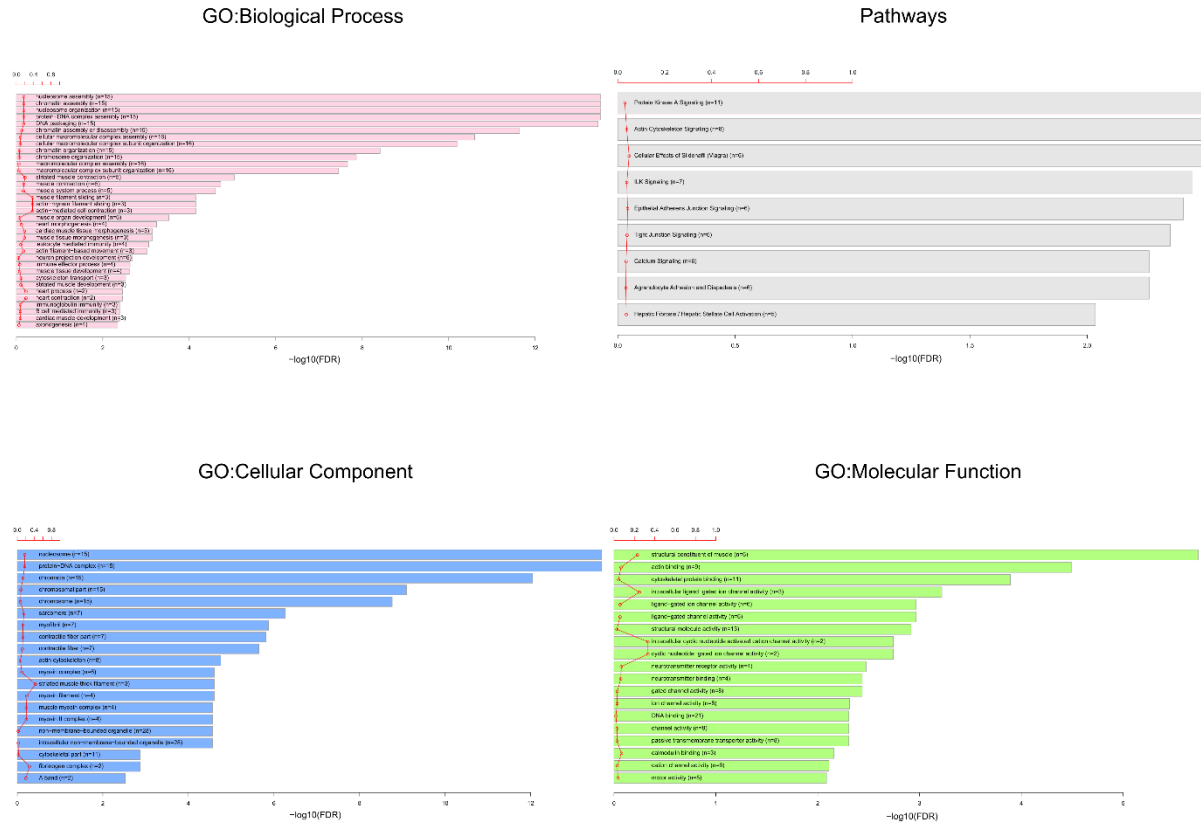
Fig 3. Gene ontology and over-represented pathways. Graphs show the canonical pathways significantly over-represented (enriched) by the DEGs (differentially expressed genes) with the number of genes for the main categories of the three ontologies (GO: biological process, GO: cellular component, and GO: molecular function, respectively). The statistically significant canonical pathways in the DEGs are listed according to their p-value corrected by FDR (- log10) (colored bars) and the ratio of the listed genes found in each pathway over the total number of genes in that pathway (ratio, red line).

These results suggest that BIRC6 could play an important role in tumor homeostasis and metastasis development. For this reason, we decided to perform a deeper analysis on apoptosis, proliferation, angiogenesis, migration and focal adhesion pathways.

i. *Apoptosis*

We evaluated the expression of Bax, Bcl-2, Caspase 9, Caspase 3, Caspase 8, TP53, Cytochrome C, NFκB1 and DIABLO under conditions of either low or high BIRC6 expression

13

256 (Fig 4A). In addition, correlation between the expression of BIRC6 and the aforementioned

257 proteins was analyzed (Fig 4B) by grouping samples according to four criteria: normal tissue,

258 tumor tissue and positive or negative hormone receptor (HR + or HR-). The results showed

259 that those samples with high expression of BIRC6 had lower expression of Bax, Caspase 9,

260 Cytochrome C and DIABLO, and higher expression of Bcl-2, Caspase 3, Caspase 8 and

261 NFκB1 when compared to samples with low BIRC6 expression. Furthermore, we observed

262 that there was no statistically significant difference in TP53 expression levels between both

263 groups (Fig 4B). Finally, we observed a differential correlation between samples of healthy

264 tissue and tumor tissue.

265
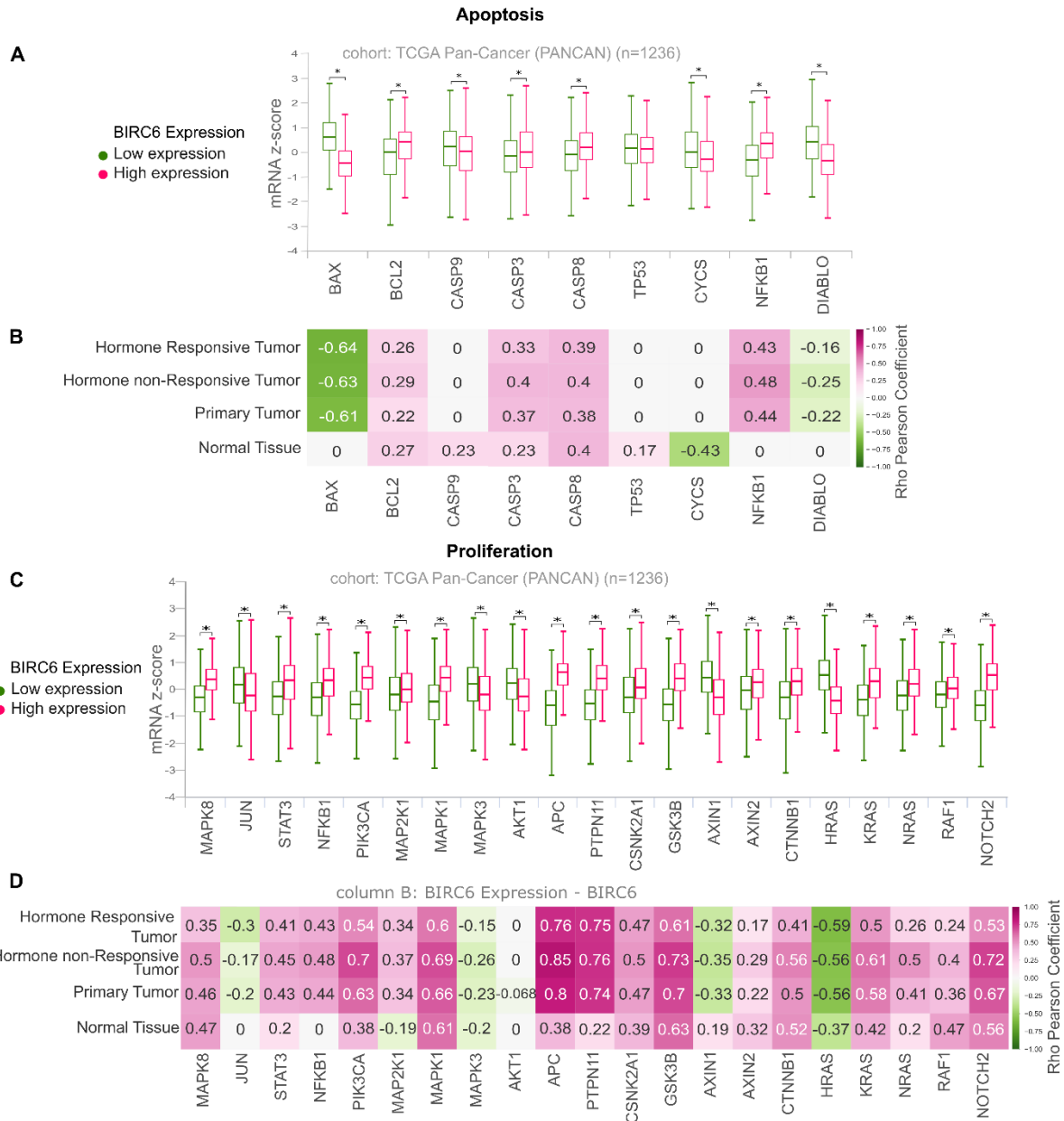
Fig 4. Transcriptomic analysis of apoptotic and proliferation pathways (TCGAPanCancer). (A) Boxplot Of The Expression (z-score) of proteins involved in the apoptotic pathway in condition of high and low BIRC6 expression. T-test *p-value<0.01. (B) Correlation between BIRC6 expression and proteins involved in the apoptotic pathway separated in: normal tissue, primary tumor, hormone responsive and hormone non responsive tumors. Numbers in cells indicate correlation pearson index in statistically significant comparisons. (C) Boxplot of the expression (z-score) of proteins involved in proliferative pathways in condition of high expression and low expression of BIRC6. T-test *p-value<0.01. (D) Correlation between BIRC6 expression and proteins involved in proliferative pathways

274   separated in normal tissue, primary tumor, hormone responsive and hormone non responsive tumors.

275   Numbers in cells indicate correlation pearson index in statistically significant comparisons.

276   **ii. *Proliferation***

277   Another essential cellular process for tumor biology is proliferation, which is exacerbated in

278   cancer cells. Taking this into account, we repeated the previous analysis using proteins linked

279   to this process. We evaluated the expression of MAPK8, JUN, STAT3, NFkB1, PIK3CA,

280   MAP2K1, MAPK1, MAPK3, AKT1, APC, PTPN11, CSNK2A1, GSK3B, AXIN1, AXIN2,

281   CTNNB1, HRAS, KRAS, NRAS, RAF1 and NOTCH2 under conditions of low and high BIRC6

282   expression (Fig 4C). In addition, the correlation between the expression of these proteins and

283   BIRC6 was analyzed in normal tissue, tumor tissue and positive or negative hormone

284   receptors samples (Fig 4D). The results showed that those samples with high expression of

285   BIRC6 have high expression of some proteins that promote proliferative pathways such as

286   STAT3, PI3KCA, MAPKs and APC.

287   ***iii. Metastatic cascade: Angiogenesis, Migration and Focal Adhesion***

288   The metastatic cascade consists of several steps: invasion, angiogenesis, surviving the

289   passage through the circulatory system, adhesion and anchorage in distant organs and,

290   finally, growth into micrometastases and, then, into consolidated metastases. We evaluated

291   the correlation between BIRC6 overexpression and key regulators of the metastatic cascade.

292   Angiogenesis is the generation of new blood vessels from pre-existing ones, which allow tumor

293   cells to spread to distant organs. We assessed the expression levels of EGFR, IGF1R, INSR,

294   HIF1A, ARNT, ANGPT, TEK and VEGFA in conditions of both low and high BIRC6 expression

295   (Fig 5A). In addition, the correlation between the expression of these proteins and BIRC6 was

296   analyzed in samples of normal tissue, tumor tissue and positive or negative hormone receptors

297   (HR + or HR-). We found that those samples with high expression of BIRC6 have high

298   expression levels of some angiogenesis promoters such as HIF1A and VEGFA (Fig 5B).

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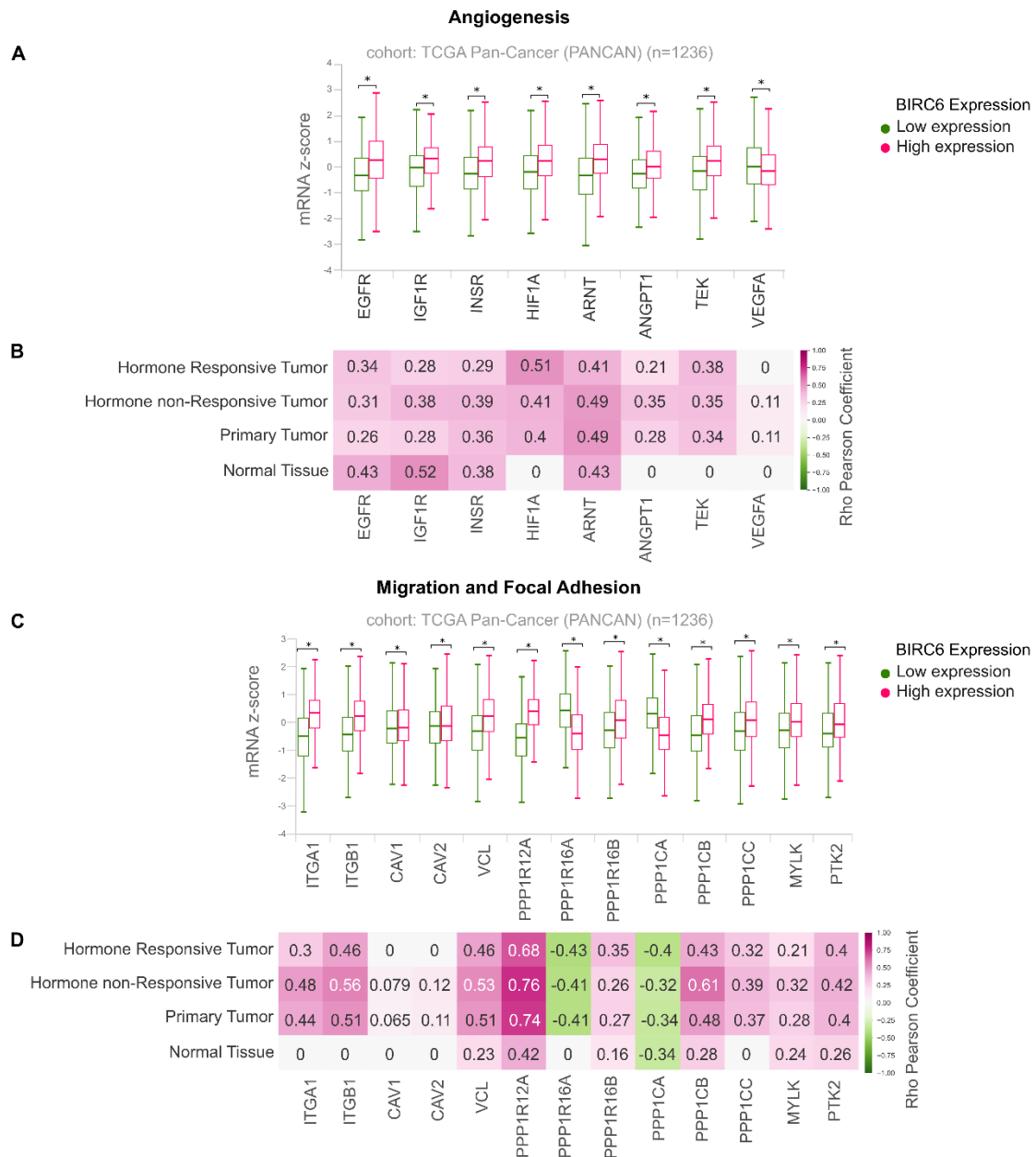
Fig 5. Transcriptomic analysis of pathways related to angiogenesis, migration and focal adhesion (TCGAPanCancer). (A) Boxplot Of The Expression (z-score) of proteins involved in angiogenesis in conditions of high and low expression of BIRC6. T-test *p-value<0.0. (B) Correlation between BIRC6 expression and proteins involved in the angiogenesis pathway separated in normal tissue, primary tumor, hormone responsive and hormone non responsive tumors. Numbers in cells indicate correlation pearson index in statistically significant comparisons. (C) Boxplot of the expression (z-score) of proteins involved in migration and focal adhesion related pathways in condition of high expression and low expression of BIRC6. T-test *p-value<0.01. (D) Correlation between BIRC6 expression and proteins involved in migration and focal adhesion related pathways separated in normal

17

309 tissue, primary tumor, hormone responsive and hormone non responsive tumors. Numbers in cells

310 indicate correlation pearson index in statistically significant comparisons.

311 We also evaluated the role of BIRC6 on migration and adhesion processes, since both are

312 necessary for tumor cells to invade other tissues and colonize distant sites, thus generating

313 metastatic nodules. We evaluated the expression of ITGA1, ITGB1, CAV1, CAV2, VCL,

314 PPP1R12A, PPP1R16A, PPP1R16B, PPP1CA, PPP1CB, PPP1CC, MYLK and PTK2 under

315 conditions of low and high BIRC6 expression (Fig 5C). We found that those samples with

316 higher BIRC6 expression presented higher expression of some of the proteins that promote

317 migration such as VCL, PPP1R12A, PPP1CB, MYLK and of those involved in focal adhesions

318 like PTK2.

319 In addition, we see a distinctive behavior regarding the correlation of BIRC6 and the different

320 proteins analyzed in tumor samples when compared to normal tissue, showing an increased

321 correlation (Fig 5D). This implies a relationship between the expression of BIRC6 and different

322 proteins involved in metastatic cascade. The complete study is presented in S2 Fig and S1

323 Table.

4. Discussion

325 Breast Cancer is the second leading cause of cancer death worldwide. With the emergence

326 of resistance to conventional therapies arises a growing trend towards the design of new

327 treatment schemes that employ specific molecular targets. Taking this into account, we set

328 out to evaluate the role of BIRC6 in BC development and metastasis by genomic and

329 transcriptomic approaches.

330 To begin the study, we analyzed BIRC6 expression in different tissues, and we found higher

331 expression levels in samples from mammary carcinomas compared to those corresponding to

332 healthy tissue. This could imply that an increase in the expression of BIRC6 could aid cells in

333 evading the physiological mechanisms of homeostasis, and subsequently become

334 carcinogenic (7,9).

335   We then assessed the correlation between different clinical attributes and the expression

336   levels of BIRC6 using databases that contained transcriptomic and genomic information of

337   patient samples.

338   The genomic analysis indicates a higher proportion of amplifications and gains in the number

339   of copies of BIRC6 in ER- and PR- samples when compared to ER+ and PR+. The loss of

340   hormone receptors results in tumors displaying much more aggressive phenotypes (46–51),

341   thus suggesting a possible relationship between BIRC6 expression levels and tumor

342   aggressiveness. We also compared the CNVs with the age of diagnosis and prognosis and

343   found that patients with amplifications or gains in BIRC6 copy number had a tendency to be

344   diagnosed at a younger age. Various studies have shown a correlation between BIRC6

345   expression and the patient's prognosis, as well as an involvement of BIRC6 in early stages of

346   cancer development, for colorectal (6), prostate (7,8), and ovarian cancer (9), among others.

347   In the light of this, the relationship of BIRC6 CNV and neoplastic histological grade and the

348   Nottingham prognostic index were analyzed (52–54). We observed that those samples with

349   amplifications or gains showed a tendency to have worse prognosis and to be in more

350   advanced neoplastic degrees. This, together with the loss of the receptors, suggests that an

351   increase in the number of BIRC6 copies could lead to early development and the generation

352   of a more aggressive BC phenotype. To conclude the genomic part of the study, we decided

353   to evaluate the CNV profile in patients who had received either chemotherapy or radiotherapy.

354   It was reported for other types of cancer that BIRC6 could participate in the mechanisms of

355   resistance to these therapies (24,27). We found that in samples of patients who had received

356   chemotherapy or radiotherapy there was a higher proportion of amplifications and gains in

357   gene copy number. Since many signaling pathways are affected in tumor physiology,

358   transcriptomic analysis of the main pathways involved were performed.

359   We evaluated the state of the different pathways under conditions of high and low BIRC6

360   expression and the correlation with the different proteins involved, in samples of hormone

361   dependent and independent mammary carcinomas. Regarding apoptosis, gene selection was

362   based on the fact that BIRC6 interacts with different caspases and SMAC/DIABLO, suggesting

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363   some kind of correlation in their expression, and also, regulating other points of the pathway

364   that are not directly related. Furthermore, Jinyu Ren et al. reported that the BIRC6 gene is

365   capable of regulating the P53 protein and the mitochondrial apoptotic pathway (15). We

366   observed that the expression of anti-apoptotic genes such as BCL-2 and NFκB1 was

367   increased in high BIRC6 expression samples and pro-apoptotic genes such as BAX, CASP9

368   and DIABLO were decreased. Despite some effectors with pro-apoptotic activity, like CASP3

369   and CASP8, correlated with BIRC6 expression, it is important to take into account that BIRC6

370   interacts directly with both of them exhibiting an inhibitory effect where the net balance results

371   in apoptosis inhibition. Interestingly, despite previous reports in which a relationship between

372   BIRC6 and TP53 was established in breast cancer (22), we found no differences in our

373   transcriptomic analysis.

374   Another mechanism involved in tumor formation is the unregulated and excessive cell

375   proliferation. It was demonstrated that BIRC6 inhibition decreases tumor cell growth in lung

376   and prostate cancer (8,24). In addition, our results demonstrate that BIRC6 overexpression

377   may promote proliferative pathways like STAT3, PI3KCA, MAPKs and APC in BC.

378   Tumor metastasis is a multistage process during which malignant cells spread from the

379   primary tumor to non-contiguous organs. The steps that lead to metastasis can be

380   summarized in a few events that are known as the metastatic cascade (55). Considering that

381   metastatic spread affects the survival and prognosis of cancer patients, each of the events

382   that are part of the metastatic cascade are attractive targets for developing therapeutic

383   strategies in oncology. In this work we studied the correlation between BIRC6 expression and

384   key regulators of the metastatic cascade. We observed BIRC6 overexpression correlated with

385   high expression of different proteins involved in migration (such as MYLK), angiogenesis

386   (HIF1A and VEGFA) and adhesion (PTK). Elevated levels of BIRC6 have been linked to cell

387   growth and to some key steps in metastasis in different types of tumors. In lung cancer, BIRC6

388   overexpression was associated with tumor progression, cell growth, colony formation,

389   migration and invasion as well as with patient metastasis stage (3). In addition, in prostate

390   cancer, the reduction of BIRC6 expression decreases cancer cell viability and proliferation

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391 (7,8). We propose that BIRC6 could be involved in tumor progression and metastatic cascade

392 in BC. However, further experiments are needed to explore our proposal *in vivo*.

393 **5. Conclusion**

394 In this work, we proposed an integrative study based on genomic and transcriptomic analyses,

395 with the aim of characterizing the role of the apoptosis inhibitor BIRC6 in Breast Cancer.

396 Genomic results show that a higher copy number of BIRC6 gene correlates with more

397 aggressive phenotypes and worse prognosis markers. Transcriptomic studies show that

398 BIRC6 is overexpressed in tumor cells and these levels strongly correlate with an antiapoptotic

399 / proliferative profile. Finally, BIRC6 could play a role in the activation of signaling pathways

400 involved in metastasis.

401 Altogether our collected data suggest that BIRC6 plays an important role in tumor homeostasis

402 and can be related with the different stages of the metastatic cascade such as angiogenesis,

403 migration and adhesion in BC (Fig 6).
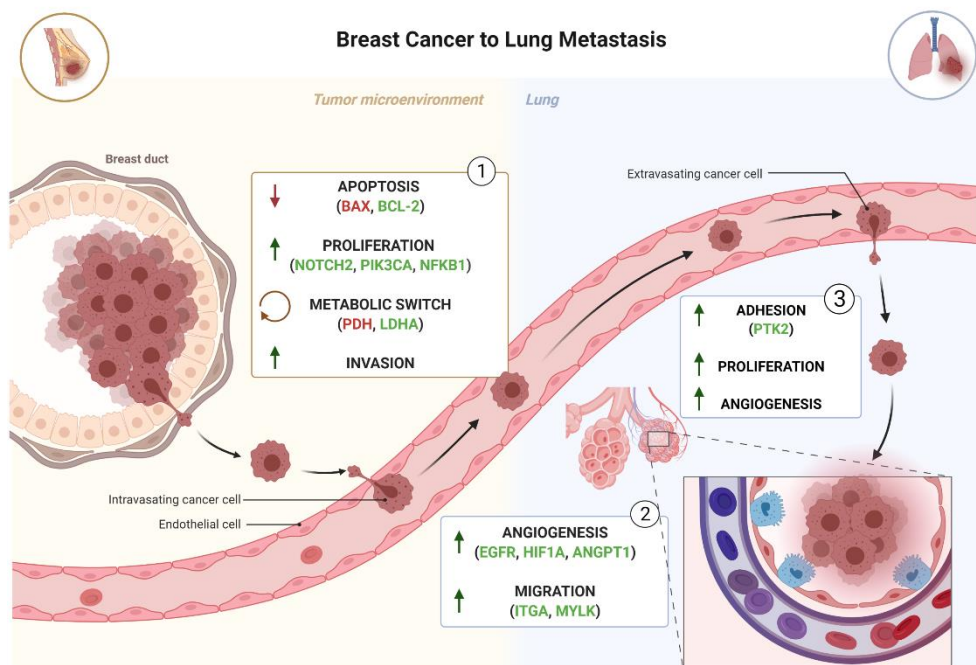
404

405 **Fig 6. Schematic representation of metastatic cascade.** Steps of metastasis are represented in three

406 groups: principal pathways in tumor microenvironment (1), pathways involved in tumor cell spreading

407   (2) and principal pathways in metastatic target organ (3). Principal genes with BIRC6 positive correlation

408   are indicated in green whilst negatively correlated are indicated in red. (Created using BioRender.com).

409   The results of *in silico* analyses presented here make it possible to postulate the inhibitor of

410   apoptosis BIRC6 as an interesting molecular target for the development of specific therapies

411   for the reduction of tumor progression and metastasis in patients with Breast Cancer.

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