Deregulated Immune Pathway Associated with Palbociclib Resistance in Preclinical Breast Cancer Models: Integrative Genomics and Transcriptomics

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Research article

Keywords: Hormone receptor-positive breast cancer, CDK4/6, drug resistance, genomics, transcriptomics, immune pathway

Posted Date: August 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-54766/v1

Version of Record: A version of this preprint was published at Genes on January 25th, 2021. See the published version at https://doi.org/10.3390/genes12020159.

Abstract

Background: Recently, cyclin-dependent kinase (CDK) 4/6 inhibitors have been widely used to treat advanced hormone receptor-positive breast cancer. Despite promising clinical outcomes, almost all patients eventually acquire resistance to CDK4/6 inhibitors. Hence, understanding the mechanisms of acquired resistance to CDK4/6 inhibitors is crucial for developing alternative treatment strategies. Therefore, the present study screened genes associated with palbociclib resistance through genomics and transcriptomics in preclinical breast cancer models.

Methods: Palbociclib-resistant cells, MCF7-PR and T47D-PR, were generated by exposing MCF7 and T47D cells to palbociclib. After confirming acquired resistance through in vitro assays, whole-exome sequencing (WES) and mRNA microarray were performed to compare the genomic and transcriptomic landscape between palbociclib-sensitive and resistant cells. Real time-PCR was performed to confirm differentially expressed genes.

Results: Microarray analysis comparing MCF7 and MCF7-PR cells revealed 651 differentially expressed genes (DEGs) (fold change >2 or <0.5), while WES comparing T47D and T47D-PR cells revealed 107 mutated genes. Furthermore, pathway analysis of both DEGs and mutated genes revealed immune pathway deregulation commonly observed in MCF7-PR and T47D-PR cells. Notably, DEG annotation revealed activation of type I interferon pathway, activation of immune checkpoint inhibitory pathway, and suppression of immune checkpoint stimulatory pathway in palbociclib-resistant cells. Moreover, mutations in NCOR1, MUC4 and MUC16 genes found in palbociclib-resistant cells were annotated to be related to the immune pathway.

Conclusions: Palbociclib resistance was found to be associated with deregulated immune pathway in preclinical breast cancer models. Further studies are warranted to evaluate whether immune pathways may be a therapeutic target to overcome CDK4/6 inhibitor resistance.

Background

Breast cancer, the most common type of cancer and one of the leading causes of mortality among women worldwide [1], can be classified into three subtypes, hormone receptor (HR)-positive, human epidermal growth factor receptor 2-positive, and triple negative breast cancer. Among the three, HR-positive breast cancer has been the most common, constituting approximately 70% of all breast cancer subtypes [2].

Cyclin-dependent kinase (CDK) 4/6 inhibitors in combination with endocrine therapy had been approved by the US Food and Drug Administration as a first-or second-line treatment of HR-positive breast cancer [3]. CDK4/6 inhibitors prevent retinoblastoma (RB) protein phosphorylation and eventually restrict G1 to S phase cell progression [4]. Despite evidence of clinical benefit, concerns regarding CDK4/6 inhibitor

resistance have been emerging [5–7]. Although several mechanisms associated with CDK4/6 inhibitor resistance, including RB loss [8], cyclin E overexpression [9], FGFR amplification [10], PTEN loss [11], and MDM2 amplification [12], had been investigated, no clinical biomarker and validated strategies for overcoming CDK4/6 inhibitor resistance have yet been available.

Immune pathways, such as immune checkpoints, have been considered imperative in cancer progression and drug resistance [13]. Various immunomodulatory cytokines, such as IL4, IL6, and TGF-β, produced by immune cells within the tumor microenvironment promote tumor growth and progression [14, 15]. Consequently, immune checkpoint inhibitors have gained attention for being one of the most promising types of immunotherapy [16]. In addition, various combinatorial strategies are currently being implemented to enhance the efficacy of immune checkpoint inhibitors [17]. For instance, studies have shown that combining immunotherapy with various chemotherapies and targeted therapies enhanced immune response and promoted enhanced checkpoint inhibitor efficacy by turning immunologically cold tumors into hot ones [17]. Furthermore, other studies have demonstrated the mechanistic association between CDK4/6 inhibition and immune response [18, 19]. In more detail, one study reported that CDK4/6 inhibition promoted T cell activation via enhancement of IL2 secretion and modulation of nuclear factor of activated T-cells activity [18]. The other study also demonstrated that CDK4/6 inhibitors altered the immune microenvironment by stimulating the production of type III interferon (IFN) and suppressing regulatory T cell proliferation [19]. Despite the enormous clinical benefits of CDK4/6 inhibitors, acquired resistance has continued to be a concern, with various studies regarding the mechanism of CDK4/6 inhibitor resistance being conducted as mentioned earlier. However, only a few studies have investigated the role of immune pathways in CDK4/6 inhibitor resistance.

Therefore, the present study generated palbociclib-resistant preclinical breast cancer models using HR + breast cancer cells and investigated mechanisms associated with palbociclib resistance through integrative genomics and transcriptomics focusing mainly on immune pathways.

Methods

Cell culture and resistant cell line establishment

HR-positive cells MCF7 and T47D were obtained from The American Type Culture Collection (Manassas, VA, USA), subsequently cultured in RPMI 1640 medium (Welgene Inc., Korea) supplemented with 10% heat-inactivated fetal bovine serum (Welgene Inc., Korea) and 1% 100x penicillin/streptomycin solution (Welgene Inc., Korea). The CDK4/6 inhibitor palbociclib was provided by Pfizer Inc. (North Peapack, NJ 07977, USA). Palbociclib-resistant cells, indicated as MCF7-PR and T47D-PR, were generated from MCF7 and T47D cells by treating with palbociclib for approximately 9 months in a stepwise dose escalating fashion (Fig 1A). Resistant cells gained a more than 10-fold higher half-maximal inhibitory concentration (IC_{50}) than their parental counterparts: 7.15 μM in MCF7-PR vs. 0.75 μM in MCF7 and 3.37 μM in T47D-PR vs. 0.26 μM in T47D-PR. Palbociclib-resistant cells also showed cross resistance to ribociclib and abemaciclib (Fig. 1B).

Gene expression via microarray analyses

Microarray analysis of palbociclib-sensitive cells and resistant cells was performed using the Affymetrix GeneChip Human 2.0 ST Array (Affymetrix, Ohio, USA). cDNAs of each sample were synthesized using the GeneChip WT (Whole Transcript) Amplification Kit according to the manufacturer's protocol. Thereafter, cDNAs were used for expression profiling using GeneChip® Hybridization, washed, and stained on a GeneChip Fluidics Station 450. The probe array was scanned using the GCS3000 Scanner (Affymetrix, Ohio, USA) and analyzed using the Affymetrix® GeneChip™ Command Console software. Data preprocessing, such as background correction, summarization, and normalization, were performed through RMA analysis in Affymetrix Power Tools.

Whole exome sequencing (WES)

To generate standard exome capture libraries, we used the Agilent SureSelect Target Enrichment protocol for the Illumina Paired-End Sequencing Library (Version C2, December, 2018) together with a 1-µg input of genomic DNA. In all cases, the SureSelect Human All Exon V6 probe set was used.

DNA quantity and quality were measured using PicoGreen and Nanodrop. Fragmentation of 1-µg genomic DNA was performed using Adaptive Focused Acoustics technology (Covaris). The fragmented DNA was repaired (i.e., an "A" was ligated to the 3' end), and Agilent adapters were then ligated to the fragments. Once ligation had been assessed, the adapter-ligated product was polymerase chain reaction (PCR) amplified. The quantity and quality of the final purified product was then determined using the TapeStation DNA screentape (Agilent). For exome capture, 250 ng of DNA library was mixed with hybridization buffers, blocking mixes, RNase block, and 5 µL of SureSelect all exon capture library according to the standard Agilent SureSelect Target Enrichment protocol. Hybridization to the capture baits was conducted at 65°C using a heated thermal cycler lid set at 105°C for 24 h on a PCR machine. The captured DNA was then amplified, after which the quantity and quality of the final purified product were determined using qPCR (according to the qPCR Quantification Protocol Guide) and TapeStation DNA screentape (Agilent), respectively. Finally, we sequenced using the NovaSeq platform (Illumina, San Diego, USA).

Sequences were aligned using BWA-0.7.12 [20] based on Genome Reference Consortium build 37 (GRCh37). After duplicate reads were removed using Picard-tools-1.130, mutations were identified using the Genome Analysis Toolkit v3.4.0 following best practice guidelines [21]. Mutation annotation was performed using four public databases [i.e., 1000 Genomes Phase 3 [22], dbSNP 142, ESP (ESP6500SI V2), and ClinVar [23] (downloaded May 2015)] and one computational prediction method [i.e., SnpEff v4 [24]]. The 1000 Genomes, dbSNP, and ESP databases were used for identifying common mutations, while clinical information regarding the pathogenicity of a mutation was determined using ClinVar. The SnpEff v2 tool was used to predict the effects of gene mutations, estimate the deleteriousness of the mutations, and classify them as "Modifier," "Low," "Moderate," and "High." For instance, large chromosomal deletions

and duplications are mutations with High impact; gene duplication has Moderate impact, intron mutations have Modifier impact; and synonymous mutations have Low impact.

Pathway analysis of DEGs

To identify DEGs, we divided the fold change in mRNA expression of the palbociclib-resistant cells by that of sensitive cells (e.g., MCF7-PR/MCF7 or T47D-PR/T47D). Gene Ontology (GO) Biology Process terms were used for pathway analysis, while the gProfileR R package (0.7.0) was used to identify statistically enriched biological processes in our DEGs. Statistical significance for pathway analysis was adjusted using the Benjamini–Hochberg method for multiple hypothesis testing correction.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's instructions. RNA was quantified and used to generate cDNA using the Takara PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc). qRT-PCR was performed using a Power-up SYBR Green Master Mix (Thermo Fisher Scientific), while mRNA detection was performed using an ABI Step One Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The comparative CT method was used to determine the relative expression in each sample using beta actin as normalized control. Primers were obtained from Macrogen (Macrogen, Inc. Korea).

Identification of driver or pathogenic mutations

To identify clinically important mutations, we initially removed common variants that had allele frequencies greater than 0.05 in the normal population and mutations with low quality (FILTER ≠ PASS). Thereafter, mutations in the coding region were selected. Lastly, pathogenic mutations were defined according to ClinVar, while mutation impact was predicted using SnpEff. Clinically important mutations, which are pathogenic or likely pathogenic in ClinVar, were identified, while High or Moderate impact mutations predicted by SnpEff were considered to be possibly pathogenic. A total of 203 clinically important mutations in 107 genes were identified in the T47D cells, while 312 mutations in 150 genes were identified in the MCF7 cells.

For the mutation dot plot, we focused on genes with greater significance than the aforementioned criteria. Firstly, we selected mutations with only High impact by removing mutations with Moderate impact. Secondly, we selected mutations in genes defined as a cancer gene by the COSMIC v87 cancer gene census (Tier 1 or 2) [25]. However, pathogenic mutations in ClinVar were not removed, although their genes were not included in COSMIC cancer gene census. Ultimately, 52 mutations in 30 genes were selected for the mutation dot plot.

Pathway analysis for genes with driver mutations by visualizing GO

The GO Biology Process terms were also used for pathway analysis of 107 and 150 genes with driver mutations in T47D and MCF7 cells, respectively. Statistically enriched biological process terms in genes with driver mutations were identified using the gProfileR R package (0.7.0). Based on the GO pathway analysis results, we then visualized enriched GO terms in our mutated genes using semantic similarity-based scatterplots in REduce and VIsulize Gene Ontology (REVIGO) [26]. To assist in interpretation, REVIGO summarizes and visualizes GO terms by finding a representative subset of terms using a simple clustering algorithm that relies on similarity measures between GO terms.

Results

DEG analysis revealing deregulation of immune pathway in palbociclib-resistant cells

To identify genes and pathways involved in the development of palbociclib resistance, microarray analysis was performed on both palbociclib-sensitive and resistant cells (Supplementary Fig. 1A, B). Comparison of gene expression between sensitive T47D and resistant T47D-PR cells identified 210 DEGs (fold change > 2 or <0.5), although no GO terms were enriched in all of them (Supplementary Fig. 1B). However, a comparison between MCF7 and MCF7-PR cells identified 651 DEGs, with GO enrichment analysis revealing that 85 genes were involved in immune pathways (Fig. 2A). Furthermore, all immune-related genes were classified into various categories according to fold changes as shown in Fig. 2B. Given the previous reports demonstrating associations between the type I IFN pathway and endocrine resistance [27] and CDK4/6 inhibitor resistance [28] in the HR+ breast cancer, a collection of type I IFN genes, such as STAT1, IRF9, and SP100, were analyzed in detail. Accordingly, type I IFN genes were found to have increased in MCF7-PR cells (Fig. 2B), which was further validated by qRT-PCR (Fig. 2C). Moreover, given the involvement of the immune checkpoint pathway in cancer progression and drug resistance [13, 29], immune checkpoint inhibitory or stimulatory genes were also analyzed in detail. Notably, our results showed that while immune checkpoint inhibitory genes such as PDL1, LAG3 and CD89 were activated, stimulatory genes such as ICOS, CD70 and CD27 were suppressed in MCF7-PR cells (Fig. 2B).

Mutation profiling revealing deregulation of immune pathway in palbociclib-resistant cells

Based on WES data, we identified 203 clinically important mutations in 107 genes from T47D or T47D-PR cells (Supplementary Table 1, Supplementary Fig. 2) and 312 mutations in 150 genes from MCF7 or MCF7-PR cells (Supplementary Table 2, Supplementary Fig. 3). Although no GO terms were significantly enriched in the 150 genes from MCF7 or MCF7-PR cells (adjusted p value > 0.25) (Supplementary Fig. 4A

and Table 1), several immune-related GO terms were significantly enriched in the 107 genes from T47D or T47D-PR cells (adjusted p value < 0.25) (Fig. 3A, Supplementary Fig 4B and Table 2). In particular, surface mucin genes, which play important roles in protecting epithelial cells and have been implicated in epithelial renewal and differentiation [30], were highly mutated in T47D-PR cells (Table 2). These mucin genes were also reported to be involved in immune regulation [31, 32].

Table 1
GO Biological Process terms related to immune responses were enriched in DEGs between MCF7-PR and MCF7 cells.

GO Biological Process	count	genes	*p*-value	adjusted *p*-value
immune response	54	ADCY5, ANG, ANXA3, BLNK, BST2, CD22, CEBPG, CLEC2D, CLU, CTSC, CTSH, CTSK, DDX58, DDX60, EGR1, EPRS, FFAR3, FRK, FTH1, GBP2, HERC5, HMOX1, IFI6, IFIH1, IFIT1, IFITM1, IGHD, IL20, IRF9, ISG15, KIR2DS2, KIR2DS4, KIR3DL2, KYNU, LYN, MYB, NFIL3, OAS1, OAS2, OAS3, PTGER4, RAET1G, RIPK2, S100A8, S100A9, SEMA3C, STAT1, SUSD2, TAB1, TXNIP, ULBP1, UNC13D, USP18, VIPR1	1.5E-10	4.3E-09
immune effector process	33	ACKR3, ANXA3, BST2, CEBPG, CLU, CTSC, CTSH, DDX58, DDX60, FFAR3, HERC5, HMOX1, IFIH1, IFIT1, IFITM1, IGHD, IRF9, ISG15, LYN, MTSS1, MYB, OAS1, OAS2, OAS3, PDK4, PTGER4, RAET1G, RIPK2, STAT1, TNIK, ULBP1, UNC13D, ZNF189	2.7E-09	6.8E-08
regulation of immune system process	45	ACKR3, ANXA1, BMP4, BST2, C5AR2, CDK6, CLEC2D, CLU, CTSH, CTSK, DDX58, DDX60, FFAR3, FLT3, HERC5, HMOX1, IFIH1, IFIT1, IFITM1, IGHD, IL20, ISG15, KIR2DS2, KIR3DL2, LYN, MITF, MTSS1, MYB, MYC, PDE5A, PDK4, PLCB1, PTGER4, RIPK2, S100A7, SHPK, STAT1, TAB1, TGFBR2, TNIK, TRIB1, ULBP1, UNC13D, USP18, ZNF189	1.4E-07	2.8E-06
innate immune response	36	ADCY5, ANG, BST2, CEBPG, CLU, CTSK, DDX58, DDX60, EGR1, EPRS, FRK, GBP2, HERC5, IFI6, IFIH1, IFIT1, IFITM1, IRF9, ISG15, KIR2DS2, KIR2DS4, KYNU, LYN, OAS1, OAS2, OAS3, RAET1G, RIPK2, S100A8, S100A9, STAT1, TAB1, TXNIP, ULBP1, UNC13D, USP18	3.5E-07	6.5E-06
immune system development	30	ANXA1, BMP4, CALCR, CDK6, CEBPG, DHRS2, EGR1, FLT3, G6PD, HERC6, IGHD, IL20, ISG15, KRT75, L3MBTL3, LYN, MITF, MPZL2, MYB, MYC, ONECUT1, PTGER4, RIPK2, RUNX2, SIX1, TGFBR2, TMOD2, TRIB1, ZFP36L2, ZNF385A	4.5E-07	8.1E-06
regulation of immune effector process	16	ACKR3, BST2, DDX58, DDX60, FFAR3, HERC5, HMOX1, IFIT1, LYN, MTSS1, MYB, PDK4, RIPK2, TNIK, UNC13D, ZNF189	0.00018	0.00182
negative regulation of immune system process	13	BMP4, BST2, C5AR2, CDK6, FLT3, HMOX1, IFIT1, LYN, MYC, PDE5A, PLCB1, PTGER4, TRIB1	0.00155	0.01199

Table 2
GO Biological Process terms related to immune responses were enriched in genes with mutations occurred in T47D cells.

GO Biological Process	count	genes	*p*-value	adjusted *p*-value
innate immune response activating cell surface receptor signaling pathway	7	MUC3A, MUC4, MUC5B, MUC6, MUC12, MUC16, MUC17	2.41E-07	7.24E-05
immune response-regulating cell surface receptor signaling pathway	8	HSP90AB1, MUC3A, MUC4, MUC5B, MUC6, MUC12, MUC17, SOS1	3.62E-05	0.0048
innate immune response-activating signal transduction	6	MUC3A, MUC4, MUC5B, MUC6, MUC12, MUC17	0.0002	0.0238
immune response-regulating signaling pathway	8	HSP90AB1, MUC3A, MUC4, MUC5B, MUC6, MUC12, MUC17, SOS1	0.0003	0.0238
immune response-activating signal transduction	7	HSP90AB1, MUC3A, MUC4, MUC5B, MUC6, MUC17, MUC12	0.0015	0.0895
regulation of immune response	8	HSP90AB1, MUC3A, MUC4, MUC5B, MUC6, MUC17, MUC12, SOS1	0.0052	0.1476
regulation of immune system process	11	HSP90AB1, KMT2C, MUC3A, MUC4, MUC5B, MUC6, MUC12, MUC17, POU4F1, RB1, SOS1	0.0086	0.1476
positive regulation of immune system process	9	HSP90AB1, MUC3A, MUC4, MUC5B, MUC6, MUC12, MUC17, POU4F1, RB1	0.0103	0.1476
immune system process	12	PRSS3, KMT2C, HSP90AB1, TGFBR1, SOS1, MUC5B, MUC4, VCP, MUC17, MUC3A, MUC6, MUC12	0.0203	0.1476

Visualization of GO revealing prominent immune process involvement in palbociclib-resistant cells

GO enriched terms in the 107 genes with mutations in T47D or T47D-PR cells (Supplementary Fig. 4B, Supplementary Table 2) were analyzed and visualized using REVIGO [26] (Fig. 3B). For a better understanding of enriched GO terms, REVIGO measures the relationship between GO terms, removes redundant terms based on similarity scores, and intuitively visualizes the representative GO term sets. When visualizing enriched GO terms, three clusters were observed. Accordingly, two clusters in the upper part of the semantic space of Fig. 3B were related to immune pathway, such as immune response and

response to stimulus, while one cluster in the lower part was related to the cancer pathway, such as cell cycle arrest, cellular senescence, and regulation of epithelial to mesenchymal transition.

Mutation dot plots

We herein identified 52 of the most clinically significant mutations in 30 genes as described in the Methods section (Supplementary Table 3, Fig. 4, and Supplementary Fig. 5). As shown in Fig. 4, MCF7-PR cells retained 29 mutations in 16 genes from MCF7 cells, among which six [BTD (G47R), FCGR3B (I142V), NBN (R43*), NPC2 (c.441+1G), PIK3CA (E545K), and SAA1 (A70V)] were reported as pathogenic in ClinVar. T47D-PR cells also retained 16 mutations in 13 genes from T47D cells, among which nine [ACTN (T716M), CYP2A6 (L160H), INSR (V1012M), NKX2-5 (E21Q), OCA2 (A481T), PIK3CA (H1047R), PRSS1 (A16V) and (N54S), and TP53 (L194F)] were pathogenic in ClinVar.

Both MCF7 and T47D cells and their palbociclib-resistant variants (MCF7-PR and T47D-PR) had mutations in PDE4DIP and PIK3CA. Interestingly, T47D-PR cells exhibited NCOR1 (R190*), MUC16 (K13558fs), and RB1 (Y659fs) mutations not found in T47D cells. In addition, MCF7-PR cells exhibited mutations in MUC4 (M3855fs, L3857fs, T3860fs, P3862fs), and RSPO2 (R64*), which were not found in MCF7 cells. The association between mutated genes found only in palbociclib-resistant cells and CDK4/6 inhibitor resistance warrants further investigation. Among the four mutated genes described above (i.e., NCOR1, MUC16, MUC4, and RSPO2), NCOR1, MUC16, and MUC4 were reported to have been directly involved in modulating tumor microenvironment and thereby mediating drug resistance [33-35].

Discussion

Little is known regarding the association between CDK4/6 inhibitor resistance and immune pathways. Using our own palbociclib-resistant preclinical model, the present study demonstrated that palbociclib resistance was associated with a deregulated immune pathway. After generating palbociclib-resistant HR-positive breast cancer cells, integrative genomics and transcriptomics were utilized to compare palbociclib-resistant breast cancer cells with their parental counterparts. Our findings may help guide future research regarding immune pathway regulation to overcome CDK4/6 inhibitor resistance.

Studies have reported that IFN signaling activation was correlated with cancer progression and emergence of drug resistance [36]. IFNs have been known to activate the JAK/STAT pathway, which promotes tumorigenesis and drug resistance via enhancement of epithelial–mesenchymal transition [36]. Mounting evidence has also indicated that genes involved in type 1 IFN signaling are involved in endocrine resistance [27, 37]. Moreover, recent reports have revealed enrichment of type 1 IFN signaling and induction of IL6/STAT3 pathway in CDK4/6 inhibitor-resistant cells [28, 38]. Similarly, the current study supported the previous results by demonstrating that a panel of type 1 IFN pathway genes, including SP100, IRF9, and STAT1, was overexpressed in palbociclib-resistant cells. This warrants further investigation on the type I IFN pathway as a potential diagnostic or therapeutic target for CDK4/6 inhibitor-resistant patients.

Based on the preclinical evidences that CDK4/6 inhibition augments immune response [18, 19] as mentioned earlier, CDK4/6 inhibitor and immunotherapy combinations have currently been evaluated in a couple clinical trials [39, 40]. Preliminary results of this phase Ib trial demonstrated that the combination of abemaciclib and pembrolizumab was safe and exhibited promising in antitumor activity [39]. This prompted us to investigate alterations in immune checkpoint genes in our palbociclib-resistant preclinical model. Interestingly, we newly found that while immune checkpoint inhibitory genes increased, immune checkpoint stimulatory genes decreased in our palbociclib-resistant preclinical model. This association between CDK4/6 inhibitor resistance and altered immune checkpoint pathway has never been previously reported. Future studies are therefore needed to obtain further insight into the combined use of CDK4/6 inhibitors and immunotherapy to overcome CDK4/6 inhibitor resistance.

Previous studies have investigated NCOR1, MUC4, and MUC16 genes for their aberrant expression in various cancers and being attractive targets for immunotherapy [35, 41, 42]. NCOR1 has been found to have an important function in proper T cell development [43]. Moreover, downregulation of NCOR1 in dendritic cells has been reported to induce FoxP3 + regulatory T cells by inhibiting Th17 cell development [35]. In addition, aberrant glycosylation of mucins interferes with the ability of natural killer cells to destroy tumor cells, suggesting immunosuppression around the tumor [32]. However, the association between mutations in such genes and CDK4/6 inhibitor resistance observed herein has never been documented thus far. Validation of our discovery regarding novel mutations in NCOR1, MUC4, and MUC16 genes would indicate that such mutations might serve as predictive biomarkers or therapeutic targets for patients with CDK4/6 inhibitor-resistant breast cancer.

Conclusions

In conclusion, after screening and analyzing various genes and their respective pathways, the present study was indeed able to determine that the immune pathway was predominantly altered in palbociclib-resistant breast cancer cells. Immune-related genes in palbociclib-resistant cells had been found to be directly or indirectly involved in modulating the tumor microenvironment, further mediating drug resistance. Collectively, our results warrant vigorous further research regarding whether immune pathways could serve as a potential target to overcome CDK4/6 inhibitors resistance.

Abbreviations

CDK4/6
cyclin dependent kinase 4 and 6
MCF7-PR
palbociclib resistant MCF7
T47D-PR
palbociclib resistant T47D
HR+
hormone receptor-positive

WES
whole-exome sequencing
RB
retinoblastoma protein
IFN
interferon
IC_{50}
half maximal inhibitory concentration
WT
Whole Transcript
qRT-PCR
quantitative real-time PCR
GO
Gene Ontology

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interests

The corresponding author, Yong Wha Moon received research funds from several pharmaceutical companies including the AstraZeneca, Eisai, Dong-A ST, Chong Kun Dang, and Celltrion. No potential conflicts of interest were disclosed by the other authors.

Funding

This work was supported by three grants: the National Research Foundation of Korea (NRF) grant

funded by the Korea government(MSIT) (No. NRF-2020R1F1A1065234), Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the ministry of Health & Welfare, Republic of Korea (grant number: HI16C1559) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. NRF-2019R1A6A1A03032888).

Authors' contributions

YWM conceived, designed and directed the project. KP was involved in the project design, carried out the experiments. SH, KP and EL analyzed the data and drafted the manuscript. NP, JH and YBC participated in the cell biology work. SKK, SAL,IS, HJA and SH reviewed the manuscript.

Acknowledgements

Palbociclib was provided by Pfizer.

Authors' information

Author and corresponding author recently received the certificate of “Top downloaded paper 2018-2019” from International Journal of Cancer. The title of the paper was “Molecular mechanisms of resistance to CDK4/6 inhibitors in breast cancer: A review”

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Figures

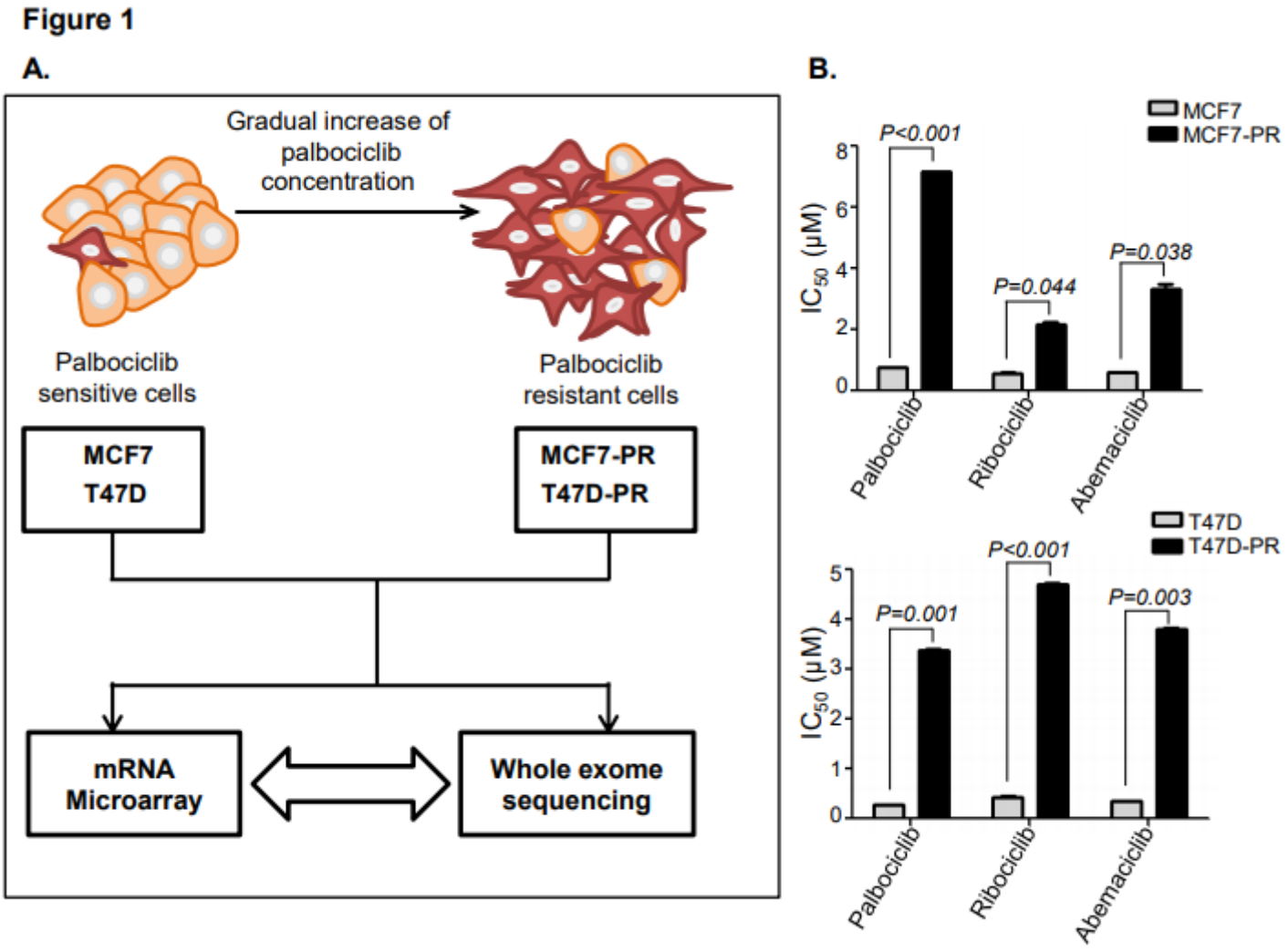

Figure 1

Schematic design of the current study. Generation of palbociclib-resistant cells. (A) Palbociclib-resistant HR-positive breast cancer cells, indicated as MCF7-PR and T47D-PR, were generated by gradually exposing MCF7 and T47D cells to increasing concentrations of palbociclib. (B) The IC50 of palbociclib in MCF7-PR and T47D-PR cells increased by around 10-fold. MCF7-PR and T47D-PR cells were cross-resistant to ribociclib and abemaciclib. P values were calculated by Student's t-test. Data are presented as means ± standard deviation of triplicate experiments. Palbociclib-resistant cells and their sensitive counterparts were compared using microarray analyses and whole-exome sequencing.

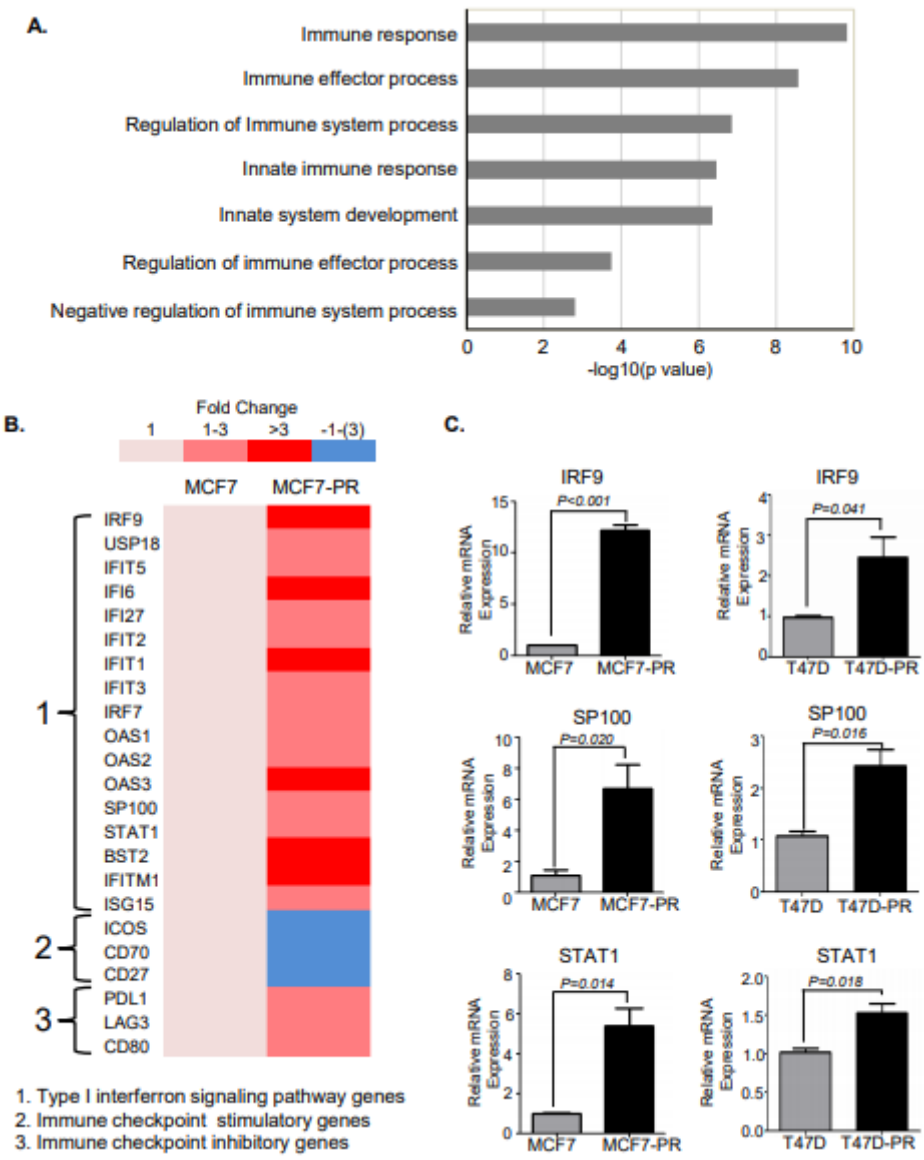

Figure 2

Differentially expressed gene (DEG) analysis in the MCF7 cells revealing immune pathway deregulation in palbociclib-resistant cells. (A) To identify DEGs, the fold change in mRNA expression of the palbociclib-resistant cells was divided by that of the sensitive cells (e.g., MCF7-PR/MCF7 or T47D-PR/T47D). Gene Ontology (GO) Biology Process terms were used for pathway analysis, while the gProfileR R package was used to identify statistically enriched biological processes in our DEGs. The bar graph shows that GO biological process terms related to immune response were enriched in DEGs. The X axis indicates p values in the specified formula −log 10 (p value). The bar represents the statistical significance of each GO term. All GO terms in Fig. 2 were statically significant (adjusted p value < 0.05). The gene list of each GO term overlapping with DEGs is provided in Table 1. (B) Panels of specific immune pathway genes are demonstrated. Type I IFN genes and immune checkpoint inhibitory/stimulatory genes were detailed in these panels to compare their expression levels between palbociclib-sensitive and resistant MCF7 cells. (C) Quantitative real-time PCR data demonstrate increased type I IFN signals. P values were calculated by Student's t-test. Data are presented as means ± standard deviation of triplicate experiments.

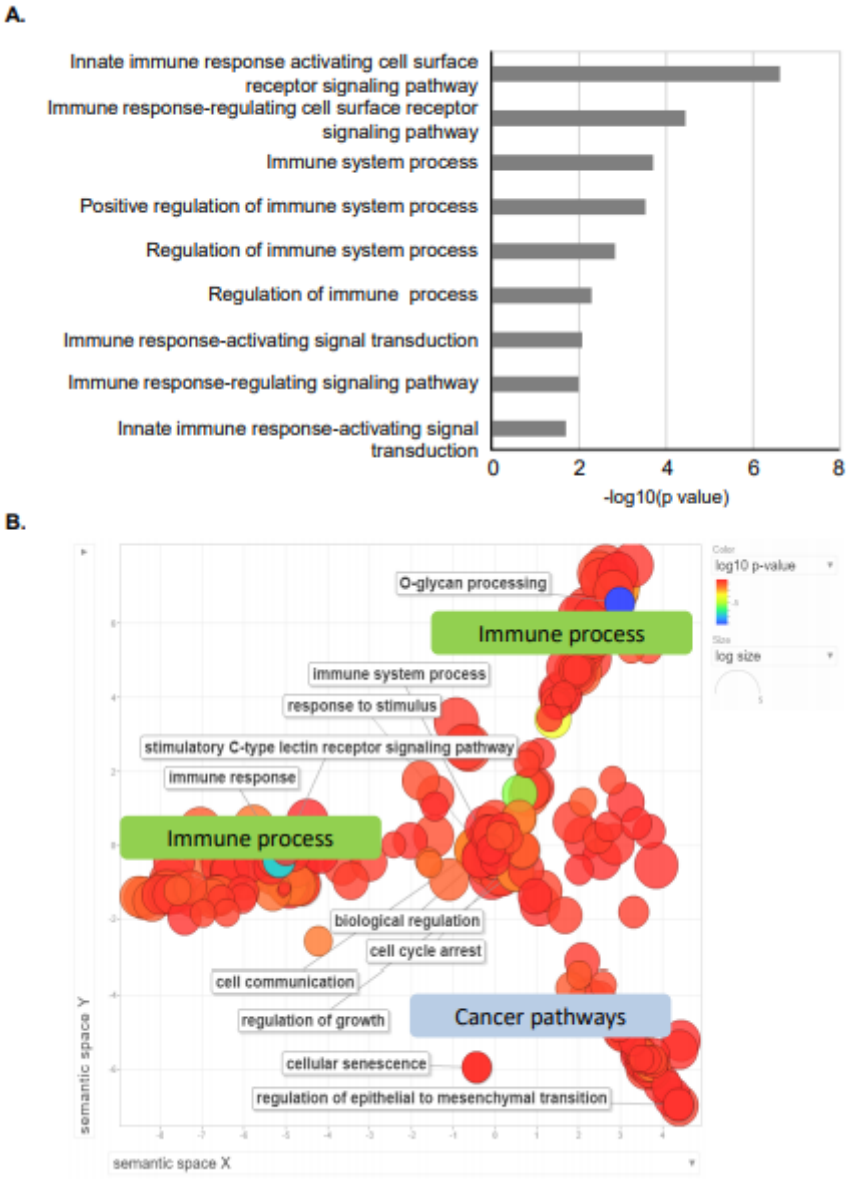

Figure 3

Mutation profiling in T47D or T47D-PR cells revealing immune pathway deregulation in palbociclib-resistant cells. (A) Mutations were identified according to the Genome Analysis Toolkit v3.4.0 following their best practice guidelines as described in the Methods section. The bar graph shows that Gene Ontology (GO) Biological Process terms related to immune response were enriched in genes with mutations occurring in T47D or T47D-PR cells. The X axis indicates p values in the specified formula −log 10 (p value). The bar represents the statistical significance of each GO term. All GO terms in Fig. 3 were statistically significant (adjusted p value < 0.25). The gene list of each GO term overlapping with genes with mutations is provided in Table 2. (B) Visualization of GO enrichment in mutated genes from T47D or T47D-PR cells revealed prominent immune process involvement in palbociclib-resistant cells. The scatterplot of representative GO terms shows the relationship between significantly enriched GO terms and genes with driver or pathogenic mutations occurring in T47D cells. The color of the circle indicates the statistical significance of the GO term in log10 of the p value. The most significant GO terms are

shown in blue, while those with the lowest significance are presented in red. The size of the circle indicates the frequency of the GO term in the GO database, with more general GO terms having a larger size. There are three clusters in the semantic space of the scatterplot. The two clusters in the upper part are related to the immune process, while the other one in the lower part is related to cancer pathways.

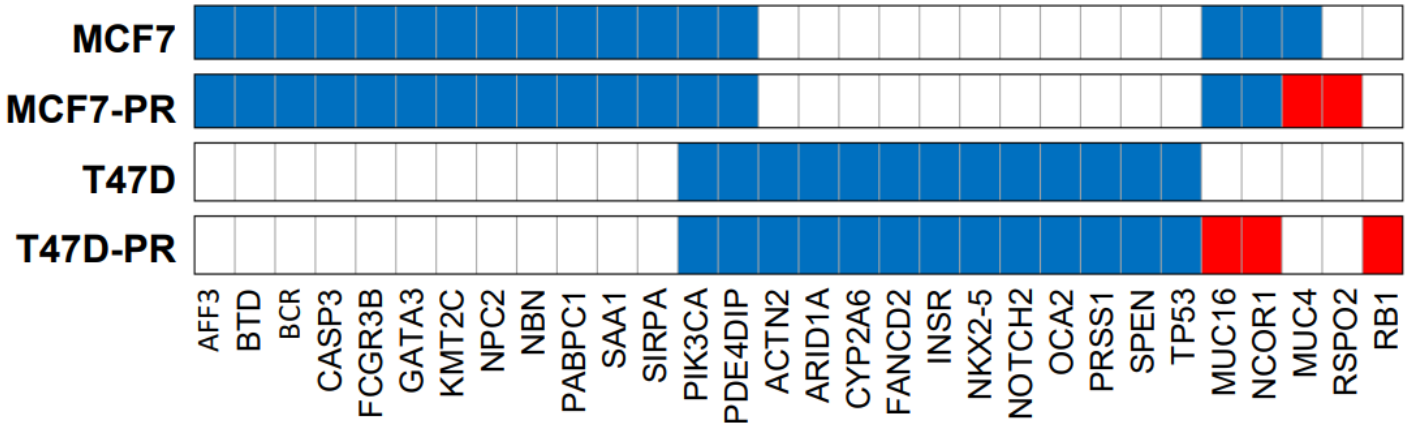

Figure 4

Mutation dot plot of 30 genes with 52 of the most clinically significant mutations. We selected mutations with only High impact or pathogenic mutations in ClinVar for the mutation dot plot. Mutations of the parent cells that remained in the resistant cells are colored blue, while newly exhibited mutations in the resistant cells are colored red. The bar graph represents the number of mutations in each cell. T47D-PR cells obtained the following new mutations: MUC16 (K13558fs), NCOR1 (R190*), and RB1 (Y659fs). Meanwhile, MCF7-PR cells exhibited the following new mutations: MUC4 (M3855fs, L3857fs, T3860fs, P3862fs) and RSPO2 (R64*).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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