Looking for biomarkers in interferon response pathway to predict response to oncolytic HSV-1 in breast cancer: An ex vivo study

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Abstract

Breast cancer is the most common malignancy in women worldwide. Administration of oncolytic viruses is one of the novel promising cancer therapy approaches. Replication of these viruses is usually limited to cancer cells that have interferon (IFN) signaling defects. However, Interferon signaling is not completely impaired in all cancer cells which may limit the benefits of virotherapy. Identification of realistic IFN-mediated biomarkers to identify patients who most likely respond to virotherapy would be helpful. In this study, eight patients-derived primary tumor cultures were infected with an ICP34.5 deleted oHSV, then the rate of infectivity, cell survival, and expression of the gene involved in IFN pathway were analyzed. Data showed that mRNA expressions of Myeloid differentiation primary response protein (Myd88) is significantly higher in tumors whose primary cultures showed less cell death and resistance to oHSV infectivity (P-value < 0.05). The differentiating cut off of Myd88 expression, inferred from the receiver operating characteristic (ROC) curve, predicted that only 13 out of 16 other patients could be sensitive to this oHSV. Identifying such biomarker improves our ability to select the patients who do not exhibit resistance to virotherapy.

Introduction

Breast cancer, the most frequent malignancy in women worldwide, is divided into 5 main subtypes based on the presence or absence of molecular markers for estrogen or progesterone receptors (ER/PR) and human epidermal growth factor (ERBB2; formerly HER2). According to these pathologic criteria, invasive ductal carcinoma (IDC) accounts for 50–75% of breast cancer cases, while invasive lobular carcinoma (ILC) accounts for 5–15% of cases. Mixed ductal/lobular carcinoma and other less common histology make up the remaining cases (1). 70–80% of patients with early-stage, non-metastatic breast cancer can be cured, while advanced breast cancer with distant organ metastases is still regarded as incurable with currently available treatments (2).

Novel breast cancer treatment concepts emphasize the need for multidisciplinary management of this heterogeneous disease and treatment based on tumor biology as well as individualization of therapy. One of the most recent developments in immunotherapy is virotherapy by oncolytic virus administration, which can selectively replicate in and kill cancer cells while causing no harm to normal tissues (3). The first effort in the design and construction of a viral genome to achieve a cancer cell-specific replicative virus was done in 1991 and resulted in a HSV-1 with a mutation in thymidine kinase (TK) for the treatment of brain tumors (4). Several mutants are currently underway in clinical trials for a variety of cancers, e.g. T-VEC (Talimogen laherparepvec, IMLYGIC, formerly OncoVEX^{GM-CSF}) has been approved by the FDA for melanoma treatment (5) while several clinical trials of T-VEC in combination with systemic administration with immune checkpoint inhibitors are ongoing.

As it appears that oncolytic virotherapy efficacy could be cell line context-dependent, and cancer cells characteristics, microenvironment, and cell signaling play a critical role in response to OVs (6). One possible candidate for such a mechanism in vivo is the interferon responses. Following viral infection, a
class of cytokines named interferon (IFNs) is produced and signals the infected and non-infected cells to prevent proliferation and downregulate metabolism, to preclude viral replication (7, 8). Type I IFN (IFNα and IFNβ) pathway is dependent on a large number of pattern recognition receptors (PRRs), the specific nucleic acid and DNA and RNA sensors, which activate the interferon-stimulated genes (ISGs) to inhibit the viral replication and limit the rate of HSV-1 infection (9). Interferon responses are impaired in the majority of cancer cells (10) and half of the silencing epigenetic changes of ISGs are linked to the immortalization of cancer cells. Indeed, deletion and lower expression of IFNs pathway genes in cancer cells promote oncolytic virus replication, which result in destroying many cancer cells, but the normal cell could be intact (11). Upregulation of several ISGs including interferon regulatory factor (IRF), myxovirus resistance (Mx), protein kinase R (PRKRA), and 2′-5′ oligoadenylate synthetase (OAS) is associated with resistance to virotherapy (12). The level of IFNs signaling impairment and the dysregulation of associated genes in the cancer cells of different patients further supports the differences in how each patient responds to virotherapy. Oncolytic virotherapy is an encouraging breast cancer therapy (13–15) and identification of realistic IFN-mediated biomarkers to target patients who are most likely responsive to virotherapy is essential. To study this, we examined the efficacy of a G207-like oHSV-1, named oHSV-RR, to infect the primary tissue culture of breast cancer patients. OHSV-RR had been constructed by Abdoli et al through the deletion of both γ34.5 copies and insertion of BleCherry coding sequence into deletion sites. (16). We then assessed the mRNA expression variation of genes related to type I IFN-pathway by qPCR assay in oHSV sensitive and resistant patient-derived primary tissue cultures. We identified at least one gene could predict the sensitivity of the patient to oHSV, and based on the calculated cut-off, we estimated that the oHSV resistance in breast cancer patients is relatively low.

Material & Methods

Sample Collection and Patient-Derived Primary Tumor Cell Culture

Twenty tumor samples and the marginal zone from the same non-diabetic luminal A and B breast cancer female patients (aged > 40 and not using immunosuppressive medication) were collected from Motamed Cancer Institute in Tehran. All procedures were performed in accordance with protocols endorsed by Zanjan University of Medical Science Ethics Committee (IR.ZUMS.REC.1397.292, and patients approved the consent form. Samples were obtained by the surgeons in aseptic condition and the lab received the samples in DMEM-Ham’s F12 supplemented with 10% Fetal Bovine Serum (FBS) and 3% penicillin/streptomycin at 4°C, immediately.

A part of the tissue was preserved in RNA later (Cat No. 1234b, Invitrogen) at -196°C and the remaining tissue was washed three times in DMEM-Ham’s F12 supplemented with 1% penicillin/streptomycin and mechanically chopped into 1–2 mm² square pieces using a scalpel in petri dish placed on ice. Tissue pieces were placed in a 15 ml centrifuge tube and digested in serum-free DMEM-Ham’s F12 containing a mixture of 1% collagenase/dispase/hyaluronidase (Cat no. SCR103, Cat no. D4693, Cat no. H1115000, 

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Sigma, respectively) in a shaking incubator (37°C, 200 rpm, 1 h). For more disintegration, the resulting mixture was gently pipetted with 0.25% (w/v) trypsin-0.53 mM EDTA. To loosen the clumping of cell suspension, the pellet was treated in DMEM-Ham's F12 containing 0.01 mg/ml DNase I. After each enzymatic disaggregation, enzymes were blocked by adding 2% FBS and discarded with the supernatant after centrifugation at 150 × g for 5 minutes. At the end, the tumor cell suspension was passed through a 100 microns (µm) cell strainer, and undigested tissue was squeezed with the sealing part of a 5 milliliters (ml) disposable syringe and the cells were collected by centrifugation at 150 × g for 5 minutes. Collected cells were seeded at a density of 5 × 10^4 cell/well onto a 24-well plate on DMEM-Ham's F12 supplemented with 10% FBS, 5 µg/ml insulin, and 1% penicillin/streptomycin (17) and incubated at 37°C in CO2 incubator. For tumor cell purification, cells were passaged with 0.05% trypsin and 0.53 mM EDTA and seeded at a density of 5 × 10^4 cells/well onto a 24-well plate.

**Virus Propagation & Titration and Ex vivo Culture Infection:**

oHSV-RR were propagated in VERO (African green monkey kidney cell) (Cat no. C101, National Cell Bank of Iran) in DMEM supplemented with 10% FBS at 37°C in CO2 incubator. 48 hour (h) post-infection, cells were scraped and lysed by freeze-thaw cycles. Then, the supernatant was collected and viruses were purified by centrifugation at 2000 g. Plaque assay was performed to calculate viral titer (18). In brief, Vero cells were seeded at a density of 3.5 × 10^5 cells/well onto a 6-well plate and infected with ten-fold serial dilution of oHSV-RR in serum-free EMEM. After 2 h incubation, media containing virus were removed and washed with PBS. 1% methylcellulose supplemented with 2% FBS was added thereafter and plates were incubated for 3 days to produce distinct plaques that are clearly countable. Methyl cellulose-overlaid was poured off and the cells were fixed using the cold 10% formaldehyde for 30 minutes, and subsequently stained with 1% crystal violet solution for 15 minutes to visualize the plaques were counted, and the average number of plaques was determined.

For confirming that the virus's ability to infect patient tumor cells is varied, oHSV-RR was added to 8 different primary tissue cultures derived from 8 patients at a multiplicity of infection of 1 (MOI 1) in a serum-free medium. Two hours after infection, oHSV-RR inoculum was discarded. Cells were washed with PBS and fresh media containing 2% FBS was replaced. At 48 h post-infection, tumor cells in a 24-well plate were observed by a CETI inverted fluorescent microscope (Medline Scientific, UK). Changes in morphology were quantified as cytopathic effect (CPE) foci while the red fluorescent dye intensity and virally infected area of every well across the whole plate area were calculated by ImageJ software (Version 1.52a, National Institutes of Health, Maryland, US).

**Cell Death Measurement By Pi Staining Flow Cytometry:**

The isolated tumor cells from primary tissue cultures were infected with oHSV-RR at MOI 1, then incubated for 48 h beside the non-infected control. All samples were detached with trypsin-0.53 mM EDTA and harvested at 400 × g for 5. The single-cell suspension was washed with PBS solution containing 0.2% FBS. The pellet was resuspended in 500 µl cold Propidium iodide (PI) buffer containing 10µg PI
(Biolegend, USA) at a density of $1 \times 10^6$ cell/ml. After 10 minutes of incubation in a dark place at room temperature, PI-stained cells were detected by flow cytometry (Attune, Thermofisher) in a 488 nm laser line. for excitation and red fluorescent for 20,000 events was measured in a BL3 channel and all results were analyzed by FlowJo-V10 software.

Rna Extraction And Rt-qpcr:

The tumor cells derived from the aforementioned 8 primary cultures onto a 24-well plate were overlaid with TRIzol (Cat no. 15596026 ThermoFisher Scientific, USA). Total RNA was isolated following the manufacturer’s protocol and treated with RNase-free Dnase. The enzyme was inactivated at 80°C for 5 minutes and the optical density (OD) 260/230 and 260/280 ratios as well as the nucleic acid concentration were measured by Epoch microplate spectrophotometer (Bio-Tek Company).

Reverse transcription was primed by oligo (dT) primers from 1 µg of total RNA and confirmed by positive control RNA available in the cDNA synthesis kit (Biotech Rabbit, Cat. No. BR0400403). The mRNA level of target genes was investigated by quantitative real time PCR while the quantification data were normalized using the housekeeping genes including GAPDH and RPLA13.

All qPCR assays were conducted in triplicated reactions on Step one plus™ real-time PCR system (Applied Biosystem) in 25µl aqueous solution containing 12.5 µl RealQ Plus 2x Master Mix Green with high ROX (AMPLIQON Cat. No. A325402), 0.1 µM of each primer and 5 µl of 1/5 diluted cDNA. The program included 1 cycle of 15 min at 95°C and 40 cycles consisting of 20” at 95°C followed by 30” at annealing temperature and 30” extension at 72°C.

The mRNA level of test was normalized to reference genes (geometric mean of GAPDH and RPL13A). Primer pairs as shown in Table 1 were designed with the IDT (Integrated DNA Technologies, Inc. USA) algorithm and Allele ID software V7.70 (Primer Biosoft) and checked for the secondary structure consideration and scoring criteria by Gene Runner V 6.5.52x64 Beta, oligo 7 software V 7.56 (Molecular Biology Insights, Inc.) and integrated DNA technologies (IDT) online tool. Finally, specific products were in-silico checked by NCBI database primer-blast and validated in vitro by gel electrophoresis of qPCR products to generate a single-size product on 2% gel and analysis of their melting curve in all experiments. The primers specific for target genes span exon-exon junctions or multi-exons separated by a large intron in qPCR. To determine the annealing temperature of the primers, gradient quantitative qPCR reactions were performed and for PCR efficiency calculation by REST software version 2.0.13 (Qiagen), a standard curve in 5 serial dilutions (500 ng/µl- 1.95 ng/µl: 1/4) was drawn on a pool of cDNA containing all samples.

**Table 1** sequence of primers designated for qPCR
### Statistical analysis

Statistical tests were performed using R software version 4.2.1 (2022-06-23 ucrt) and SPSS version 26. P-values < 0.05 was considered statistically significant. The results were expressed as mean ± SEM.

The ratio of PI positive cells was measured in each patient-derived primary tissue culture. An arbitrary cut off of 10% survival compared to the untreated corresponding sample was used to define sensitive tumors. Unpaired T-test with the assumption of variance inequality was then used to compare the variables in resistant and sensitive groups. A P-value less than or equal to 0.05 is considered statistically significant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5´ → 3´</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR9</td>
<td>F:ATCCTTCCCTGTAGCTGCTGTC</td>
</tr>
<tr>
<td>NLRP3</td>
<td>F:AAGAGGAGTGGATGGGTTTAC</td>
</tr>
<tr>
<td>MX1</td>
<td>F:GGTATAAGATCAAGACACTCAT</td>
</tr>
<tr>
<td>STAT3</td>
<td>F:ATTTAACATTCTGGGCACAACACAA</td>
</tr>
<tr>
<td>STING</td>
<td>F:GGCTGGGCATGGTCATATTACATCGGA</td>
</tr>
<tr>
<td>PRKRA</td>
<td>R:CGCTCAGTTCATCTATATCC</td>
</tr>
<tr>
<td>IRF3</td>
<td>F: TCGTGATGGTCAAGGTTGT</td>
</tr>
<tr>
<td></td>
<td>R: GGTGGCTGTTGGAAATGTG</td>
</tr>
<tr>
<td>MyD88</td>
<td>F: GGTGGTTGTCTCTGATGATTACC</td>
</tr>
<tr>
<td></td>
<td>R: GCGAGTCCAGAACAAGATTG</td>
</tr>
<tr>
<td>IFNG</td>
<td>F: ACCAGAGCATCCAAAAGAGTG</td>
</tr>
<tr>
<td></td>
<td>R: GTATTGCTTTGCGTTGGACA</td>
</tr>
<tr>
<td>OAS1</td>
<td>F: AAGCTCAAGAGCCTCATCCG</td>
</tr>
<tr>
<td></td>
<td>R: GCAGAGTTGCTGGTAGTTTATGA</td>
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<tr>
<td>GAPDH</td>
<td>F: TGGTATCGTGGGAAGGACTCATGAC</td>
</tr>
<tr>
<td></td>
<td>R: ATGCCAGTGAGCTTCCCGTTCAGC</td>
</tr>
<tr>
<td>RPL13-A</td>
<td>F: GAGGCCCTACCACCTTCC</td>
</tr>
<tr>
<td></td>
<td>R: AACACCTTGAGACGGTCCAG</td>
</tr>
</tbody>
</table>
The predictive power of genes was tested by area under the receiver operating characteristic (ROC) curve analysis (AUC, 95% confidence interval). The best cut off for discrimination between resistant and sensitive samples was also determined by ROC curve analysis.

Results

Patient-derived tissue culture of luminal samples:

24 breast tumor samples (20 fresh and 4 cryopreserved sample) were categorized into 2 groups according to hormone receptor status: luminal A ER+/PR+/HER2- (n = 18), and luminal B ER+/PR+/HER2+ (n = 6). All tumor samples were diagnosed with the invasive ductal carcinoma (IDC) type which 80% were described as low grade (grade ≤ 2) and the remaining were described as high grade. The mean age of patients was 49.9 years (standard deviation = 8.1). 8 breast samples from 20 fresh samples (culturing efficiency 40%) were successfully dissociated into primary tissue culture (Fig. 1). All tumors whose primary culture were succeed, showed low grade (grade ≤ 2) profile and 87.5% were luminal A (n = 7) and 12.5% were luminal B (n = 1). All the normal like tissue cultures serving as intrapatient control were failed.

Differential Sensitivity Of Tumor Cells Infected With Ohsv-rr:

To assess the sensitivity of patient-derived primary tissue culture to oHSV-RR, the ability of the virus to make CPE and the red fluorescent dye intensity of BleCherry were microscopically monitored 48 h post-infection. The result showed that primary cultures derived from four patients (we named sensitive group) were approximately obliterated 48 h post infection but the resistance for four other patients (we named resistant group) showed no overt CPE. (Fig. 2a-d)

The comparison of mean scores for red fluorescent intensity as analyzed by ImageJ software reveals that oHSV1-RR infection and the virally infected area of every well across the whole tumor cell culture area in primary tissue culture derived from four patients’ numbers (sensitive group) is significantly higher than four other patients (resistant group) (P-value < 0.05) (Fig. 2e-f)

The correlation between dead cell ratio in flow cytometry and BleCherry expression in fluorescent microscopic examination of tissue cultures

Flow cytometry data analysis by FlowJo-V10 software was concordant with the microscopic observation data showing different sensitivity to oHSV-RR. 48 h post-infection, PI positive population of oHSV-RR infected at MOI 1 was subtracted from PI positive population of non-infected control cells (Fig. 3a-b). Data has shown that the mean percentage of PI-stained cells of primary cultures derived from four sensitive patients is 6 times higher than the other four resistant patients (Unpaired T-test, P-value < 0.05). (Fig. 3c)
The role of the interferon pathway in the resistance of patient-derived primary culture to oHSV-RR infection:

Among the studied interferon pathway genes only the expression of Myeloid differentiation primary response protein (MyD88) was distinctively elevated in original biopsies of patients whose primary tissue cultures were resistant to oHSV-RR. (P-value = 0.033) (Fig. 4). (Two Sample T-test Analysis).

Besides, there was no significant difference between the expression levels of the studied genes (normalized to GAPDH and RPL13A housekeeping genes) in the non-infected primary tissue culture compared to the same gene in the tissue biopsy samples indicating that the expression of these genes were not affected by culturing (Two Sample T-test) (Fig. 5).

There was also no significant change in the expression level of the targeted genes post infection in patient-derived primary tissue culture that showed resistance to oHSV-RR compared to the primary tissue cultures in the sensitive group (Two Sample T-test) (Fig. 6).

Discussion

Our data show a significant difference in MyD88 gene expression in breast cancer biopsy between patients whose tumors primary cultures exhibit resistance or sensitivity to oHSV-RR infection. Indeed, our results show the variability of breast cancer patients in responding to virotherapy and proposes MyD88 as a biomarker to predict whether patients may benefit from virotherapy. Two Sample T-test analysis indicated that MyD88 mRNA expression in the original biopsy of resistant patient-derived primary tissue cultures was significantly higher than the sensitive patient-derived primary tissue cultures (P-value = 0.033). At the same time, ROC curve was exploited to evaluate the performance of a test based on this gene to distinct between sensitive and resistant patients. This analysis indicates that MyD88 has a good potential to predict the resistance to oHSV-RR. The area under curve (AUC) is 0.94 which representing high accuracy (Fig. 7).

We have studied the fold increase ratio of targeted genes expression in these twenty fresh tumor and four cryopreserved samples. The ROC curve indicates that a patient with the expression level ratio of Myd88 to reference genes (geometric mean of GAPDH and RPL13A) of more than the cut off 283.9 is most likely resistant to oHSV-RR virotherapy (sensitivity 100%, specificity 75%), as shown in Fig. 7. Applying this cut off on the 16 not-cultured samples indicated that MyD88 expression in one tumor sample is highly more than cut off (766.6) (Fig. 10), while 2 others are very close this cut off. As a result, 81 percent’s (13 out of 16) of patients of this study, may benefit from virotherapy and 6–19 percent probably may not. It is noteworthy that both the culture process and viral treatment of the sensitive samples mildly increased the expression of Myd88, however this increase is not comparable with hundred folds difference between the original biopsies of two groups. This may further support the hypothesis that the mechanism of inducing resistance to oHSV-1 is related to high primary level of MyD88 in the tumor cells (Fig. 5, 6).
Type I IFN signaling pathway is a crucial aspect of innate immunity that modifies the intracellular environment by activating a network of ISGs encoding proteins with antiviral, anti-proliferative, and immunomodulatory effects and signals the presence of a viral pathogen to the adaptive immune response (19). MyD88 is an intermediary molecule that is recruited by toll-like receptors (TLRs). HSV-1 nucleic acid is detected by TLR9 and downstream protein MyD88 is recruited via the toll/IL-1 receptor (TIR) domain. TLR9-MyD88 acts as a primary DNA sensing mechanism and initiates a downstream signaling cascade terminated by NF-κB-mediated rapid inflammation that is characterized by increased expression of a number of interleukins and cytokines and subsequently traffics to lysosome-related organelles (LRO) to induce type I IFNs. MyD88 is also involved in the formation of discrete protein complexes to activate interferon regulatory factor 3 (IRF3) and IRF7 for the expression of IFNβ (20–23).

Myd88 is employed by almost all TLRs and activated against a broad class of pathogen-associated molecular patterns (PAMP) including RNA, DNA, and lipopolysaccharide (LPS) and the critical role of this key protein to control the pathogen infection is reported previously (24, 25).

Numerous findings confirm that the different genes involved in the IFN pathway mediate the resistance to virotherapy to various types of oncolytic viruses. It seems that these inconsistent outcomes depend on the type of cell, oncolytic virus, root of administration, and other factors. In 2012, Grdzelishvili et al. analyzed the expression of 33 genes associated with type I IFN pathways, including IRF3, PRKRA, MxA, and OAS, in a panel of 11 human PDA (Pancreatic Ductal Adenocarcinoma) cell lines in response to different viruses. The type I IFN signaling status of PDA cells was shown to be heterogeneous, and MxA and OAS are suggested as potential biomarkers for resistance to VSV in highly resistant PDA cells. However, HSV and vaccinia virus's potential to infect and kill PDA cells (unlike VSV) did not depend on the type I IFN signaling (26). Wenmin Fu et al. in 2021 (27) also found that Mx2 protein mediates tumor resistance to oHSV-1 infection in human glioblastoma cell line A172 which intrinsically accumulated this protein at the highest levels. Likewise, it was found that MyD88 mRNA levels in A172 cells fell as a result of Mx2 depletion and Mx2 knocking down. IFN production, IRF3 activity, and NF-κB signaling decrease subsequently, which to increased virus yield in A172 cells.

Except for Myd88, our finding does not support the significant difference in other targeted genes like Mx1 (P-value = 0.08) between sensitive and resistant samples (Fig. 5). However, the area under curve (AUC) for Mx1, PRKRA and STING is 0.94 which is suggestive for high predictive potential of these three genes as well (Fig. 8). However, the statistical power of this study is not high enough to exclude the possibility of becoming statically significant, if the number of cultures derived from different patient was higher. This may also find additional biomarkers to improve the prediction of resistance to virotherapy with oHSV-1. In general, the main culture's success rate is typically poor (28). We succeeded in establishing eight primary cultures from 20 fresh tumors (an efficiency of about 40%). The receipt of tissue mass containing a sizable number of live cells obtained during tumor dissociation (depending upon initial tumor size and cellularity), the tumor origin (primary tumor or metastatic), and the presence of tumor stem cells are just a few of the requirements for successful tissue culture (29). The live cell yield underlines the complexity and diversity of patient-derived tissue culture data collection because it might have limited the RNA
extraction yield and subsequent assays throughput, and the number of different MOIs of oHSV-1 used for cell infection. Cell growth confluency is a significant source of uncertainty in the results of several tests like proliferation assays which might have been affected by the non-uniform growth pattern of the cells in primary culture.

Taken together, we have, for the first time, provided the experimental evidence of an IFN pathway-depended gene related to resistance to virotherapy with an oHSV-1 in breast cancer patient-derived tissue culture which more closely resembles the patient tissue in vivo than the established breast cell lines used in previous studies. Our data show that the mRNA level of target genes in patient-derived primary cultures did not significantly differ from their biopsy tissues (P-value > 0.05) indicating that culturing did not change the patients’ gene expression status (Fig. 5). As a result, this ex vivo model is well correlated to original tissue and has a clear advantage over cell culture and other in vitro models in predicting the response of patients to virotherapy. We also show that the expression level of target genes normalized to reference genes in patient-derived primary cultures do not significantly change after viral infection (Fig. 6).

Patient-derived culture in 3D model is a more promising approach which can mimic the complexities of tumor more than monolayer culture system. The tumor microenvironment is complex and the core (center) of tumor is less oxygenated and more acidic. It seems that 3D models can mimic these condition and affect the HSV-1 replication and induce resistance to virotherapy, consequently (30). In silico analysis recently has shown the networks of differentially expression genes as potential hypoxia biomarkers which may affect the oncolytic virus replication too (Shayan et al, in-press). mRNA profiling of such experimental models through RNASeq and combination of OVs with IFNs modulators can identify more (IFN-dependent/independent) biomarkers involved in resistance to virotherapy and promote our understanding for personalized medicine.

**Conclusion**

Breast cancer is not a homogenous disease but a diverse set of diseases with heterogeneity in protein expression. This heterogeneity alters patients’ response to virotherapy and proves the need for biomarkers to predict how each patient will react to the therapy.

A deeper understanding of the IFN pathway dependent/independent key players to determine the viral sensitivity of the patients’ tumor cells to OV is vital for the selection of only those patients who are more likely to benefit from OV treatment to decrease the costs and side effects of treatment. The markers inducing OV resistance give us new insights regarding the effectiveness of combinatorial therapy approaches to overrule those resistance genes and construct a new generation of gene-manipulated OVs that override the genes involved in virotherapy resistance too.

**Declarations**
Ethical Approval:

All procedures were performed in accordance with protocols endorsed by Zanjan University of Medical Science Ethics Committee (IR.ZUMS.REC.1397.292), and patients approved the consent form.

Competing Interests:

The Authors declares that they have no any competing interest to disclose.

Author Contribution:

Zahra Nejatipour: wrote the manuscript text and did all experiments

Mahdieh Shokrollahi Barough: compartment in experiments

Ladan Teimouri Toulabi, Ramin Sarrami Forooshani: patient sample supplier and reviewed the manuscript

Mohammad Farahmand: Statistical Analysis

Alireza Biglari, Kayhan Azadmanesh: Correspond Author, reviewed the manuscript

Funding:

Zanjan University of Medical Science

Availability of Data and Materials:

All dataset could be accessed. But any transfer of the patients’ samples needs authorization by the national ethical board of the ministry of health.

References


Figures

**Figure 1**
Primary tumor cell culture derived from a patient. a) after 5 h and b) after 24 h

Figure 2

Different sensitivity of tumor cells derived various patients to oHSV-RR infection. A sensitive primary tissue culture by light vision (a) and UV vision (c). A resistant primary tissue culture by light vision (b) and
UV vision (d). The mean score of image c (micrograph e) is significantly higher than image d (micrograph f) (P-value<0.05)

Figure 3

Different dead tumor cell ratio of various patients following oHSV-RR infection by Flow cytometry. The PI-positive population of oHSV-RR infected subtracted from non-infected control culture in a sensitive tumor (a) in a resistant tumor (b). Unpaired T-test shows that the mean percentage of PI-stained cells of primary cultures derived from four sensitive patients is 6 times higher than the other four resistant patients (c) (P-value <0.05).
Figure 4

The higher expression of MyD88 in tissue whose primary tissue cultures were resistant to oHSV-RR Two-Sample T-test analysis shows that the mRNA fold increase ratio of of MyD88 expression in the origin tissues of resistant primary tissue cultures is statically significantly higher than in the sensitive group (P-Value ≤ 0.05).
No significant change in gene expression during culturing of tumor cells. Two Sample T-test indicates that there is no significant difference between the mRNA levels in the primary tissue culture normalized to GAPDH and RPL13A mRNA levels in comparison with the same in the tissue sample (P-value > 0.05)
Figure 6

No significant change in resistant primary tissue cultures gene expression compared to sensitive group. Two-Sample T-test Analysis indicated that the expression level of targeted genes in resistant primary tissue cultures does not significantly differ from the sensitive primary tissue cultures.
Figure 7

Receiver operating characteristic (ROC) of MyD88. The value of area under curve (AUC), with 95% confidence interval for diagnostic experiment. AUC= 0.94 indicated a higher diagnostic accuracy.

Figure 8
ROC of Mx1, STING, and PRKRA. The value of area under curve (AUC), with 95% confidence interval for diagnostic experiment. AUC= 0.94 for Mx1 (a), STING (b), and PRKRA (c) indicated a high diagnostic accuracy.