УДК 617.51/.53-006.61:615.849.12
https://doi.org/10.20538/1682-0363-2024-1-37-47

CC BY 4.0

# Study of head and neck squamous cell carcinoma transcriptome after proton therapy

**Jumaniyazova E.D.[1], Vishnyakova P.A.[1,2], Chirkova M.V.[3], Karpulevich E.A.[3], Eremina I.Z.[1], Gordon K.B.[1,4], Kaprin A.D.[1,5], Fatkhudinov T.H.[1,6]**

*[1] Research Institute of Molecular and Cellular Medicine of the Peoples' Friendship University of Russia (RUDN Research Institute of MCM)*
*6, Miklukho-Maklaya Str., Moscow, 117198, Russian Federation*

*[2] National Medical Research Center of Obstetrics, Gynecology and Perinatology named after Academician V.I. Kulakov*
*4, Akademika Oparina Str., Moscow, 117997, Russian Federation*

*[3] Ivannikov Institute for System Programming of the Russian Academy of Sciences (ISP RAS)*
*25, Aleksandra Solzhenitsyna Str., Moscow, 109004, Russian Federation*

*[4] A. Tsyb Medical Radiological Research Center – branch of FGBU National Medical Research Center of Radiology (a branch of FGBU National Medical Research Center of Radiology)*
*10, Marshala Zhukova Str., Obninsk, 249036, Russian Federation*

*[5] National Medical Research Center of Radiology (NMC Radiology of the Ministry)*
*4, Koroleva Str., Obninsk, 249036, Russian Federation*

*[6] Avtsyn Research Institute of Human Morphology*
*3, Tsyurupy Str., Moscow, 117418, Russian Federation*

## ABSTRACT

**Aim.** To evaluate changes in the transcriptome of head and neck squamous cell carcinoma (HNSCC) tissue cells in patients after proton therapy.

**Materials and methods.** Biopsy material obtained from 3 HNSCC patients before and after proton therapy at a total dose of 10 isoGy was homogenized, purified, and concentrated. Then total RNA was isolated with further purification and concentration with the RNA Clean & Concentrator kit (Zymo Research). Library quantitation was assessed using the Qubit 2.0 instrument (Invitrogen, Life Technologies). After isolation of 1 μg total RNA for sequencing, libraries were prepared on the Illumina platform using the TruSeq RNA Sample Prep Kit v2 with a 10-cycle enrichment step according to the manufacturer's recommendations. The quality of RNA and the resulting libraries was checked using the Agilent 2100 Bioanalyzer system (Agilent Tec. Inc., USA). The RIN parameter for RNA was at least 7. The library concentration was assessed by real-time PCR on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Final libraries were pooled in equimolar ratios before sequencing on the Illumina HiSeq 2500 platform using 50 base-pair paired-end reads. The Q20 parameter for all samples was > 97%, and the number of reads averaged 60.2 million per sample. Raw reads were processed using the RTA 1.17.21.3 and Casava 1.8.2 (Illumina). The enrichment analysis was performed using the PANTHER 17.0 software.

**Results.** The transcriptome analysis of HNSCC after proton radiation therapy (5 x 2 isoGy) at a total dose of 10 isoGy revealed 1,414 significantly differentially expressed genes. The 10 most and least expressed genes and their associated signaling pathways were identified. A number of signaling pathways associated with the underexpressed genes were detected in HNSCC after proton therapy, such as: STAT5; PD-1 signaling pathway; marked MET-mediated activation of PTK2 signaling pathway, PDGF signaling; CD22-mediated regulation of

✉ *Jumaniyazova Enar D.,* enar2017@yandex.ru

BCR; and FCERI-mediated MAPK activation. In addition to the above signaling pathways, activation of collagen degradation, FCGR3A-mediated phagocytosis, and FCGR3A-mediated interleukin (IL)-10 synthesis are of interest. In the enrichment analysis among highly expressed genes, keratinization and biological oxidation processes were activated in HNSCC tissues after proton therapy.

**Conclusion.** Proton therapy in HNSCC leads to overexpression of genes involved in the regulation of keratinization and biological oxidation processes as well as to underexpression of genes associated with suppression of signaling pathways: STAT5, PD-1, MET-mediated activation of PTK2 signaling pathway, PDGF signaling; CD22-mediated regulation of BCR; FCERI-mediated MAPK activation, collagen degradation, FCGR3A-mediated phagocytosis activation, and FCGR3A-mediated IL-10 synthesis. All signaling pathways of underexpressed genes function in HNSCC cells if there is no negative influence on the tumor from outside (irradiation or delivery of antitumor drugs). The predominance of suppressed signaling pathways over activated ones most likely indicates a decrease in the functional potential of cells after proton therapy. The dose-dependence of proton therapy effects requires further study of changes in cellular and molecular-genetic signatures of HNSCC after proton therapy at different doses.

**Keywords:** head and neck squamous cell carcinoma, transcriptome, proton therapy, signaling pathways

**Conflict of interest.** The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

**Source of financing.** The study was supported by the Ministry of Science and Higher Education of the Russian Federation (agreement No. 075-15-2022-294 dated April 15, 2022) and the Ministry of Science and Higher Education of the Russian Federation, agreement No. 075-15-2021-1356 dated October 7, 2021 (identifier RF 0951.61321X0012, No. 15.SIN.21.0011).

**Conformity with the principles of ethics.** All patients signed an informed consent to participate in the study. Before enrollment in the study, the study protocol and patient information and informed consent forms were approved by an independent Ethics Committee (Protocol No. 634 of 17.11.2021, Protocol No. 684 of 02.03.2022).

**For citation:** Jumaniyazova E.D., Vishnyakova P.A., Chirkova M.V., Karpulevich E.A., Eremina I.Z., Gordon K.B., Kaprin A.D., Fatkhudinov T.H. Study of head and neck squamous cell carcinoma transcriptome after proton therapy. *Bulletin of Siberian Medicine.* 2024;23(1):37–47. https://doi.org/10.20538/1682-0363-2024-1-37-47.

# Исследование транскриптома плоскоклеточного рака головы и шеи после протонного облучения

**Джуманиязова Э.Д.[1], Вишнякова П.А.[1, 2], Чиркова М.В.[3], Карпулевич Е.А.[3], Еремина И.З.[1], Гордон К.Б.[1, 4], Каприн А.Д.[1, 5], Фатхудинов Т.Х.[1, 6]**

*[1] Научно-исследовательский институт молекулярной и клеточной медицины Российского университета дружбы народов (НИИ МКМ РУДН)*
*Россия, 117198, г. Москва, ул. Миклухо-Маклая, 6*

*[2] Национальный медицинский исследовательский центр акушерства, гинекологии и перинатологии (НМИЦ АПГ) им. акад. В.И. Кулакова*
*Россия, 117997, г. Москва, ул. Академика Опарина, 4*

*[3] Институт системного программирования им. В.П. Иванникова Российской академии наук (ИСП РАН)*
*Россия, 109004, г. Москва, ул. Александра Солженицына, 25*

*[4] Медицинский радиологический научный центр (МРНЦ) им. А. Цыба – филиал Национального медицинского исследовательского центра радиологии (филиал «НМИЦ радиологии»)*
*Россия, 249036, г. Обнинск, ул. Маршала Жукова, 10*

*[5] Национальный медицинский исследовательский центр (НМИЦ) радиологии*
*Россия, 249036, г. Обнинск, ул. Королева, 4*

*[6] Научно-исследовательский институт морфологии человека (НИИМЧ) им. акад. А.П. Авцына*
*Россия, 117418, г. Москва, ул. Цюрупы, 3*

## РЕЗЮМЕ

**Цель** – оценить изменения транскриптома клеток ткани плоскоклеточного рака головы и шеи (ПРГШ) у пациентов после протонного облучения.

**Материалы и методы.** Биопсийный материал, полученный от трех пациентов ПРГШ до и после протонного облучения в суммарной дозе 10 изоГр, был подвергнут гомогенизации, очистке и концентрации. После чего была выделена тотальная РНК с последующей очисткой и концентрацией набором RNA Clean & Concentrator (Zymo Research), количество оценивали с помощью прибора Qubit 2.0 (Invitrogen, Life Technologies). После выделения тотальной РНК из 1 мкг для секвенирования на платформе Illumina были приготовлены библиотеки с использованием набора TruSeq RNA Sample Prep Kit v2 с этапом обогащения в 10 циклов в соответствии с рекомендациями производителя. Качество РНК и полученных библиотек проверялось с помощью системы капиллярного электрофореза Agilent 2100 Bioanalyzer (Agilent Tec. Inc., США). Параметр RIN для РНК составлял не менее 7. Концентрацию библиотек оценивали с помощью полимеразной цепной реакции в реальном времени на приборе CFX96 Touch Real-Time PCR Detection System (Bio-Rad, США). Окончательные библиотеки объединяли в эквимолярных пропорциях перед секвенированием на платформе Illumina HiSeq 2500 с использованием парно-концевых прочтений по 50 оснований. Параметр Q20 для всех образцов составил более 97%, а количество прочтений в среднем равнялось 60,2 млн на образец. Сырые прочтения были обработаны с использованием RTA 1.17.21.3 и Casava 1.8.2 (Illumina). Анализ обогащения был выполнен с помощью программного обеспечения PANTHER 17.0.

**Результаты.** В ходе транскриптомного анализа ПРГШ после пятикратного облучения пациентов протонами (2 изоГр) в суммарной дозе 10 изоГр было обнаружено 1 414 значимо дифференциально экспрессированных генов. Выделены 10 наиболее и наименее экспрессируемых генов и ассоциированные с ними сигнальные пути. В ПРГШ после облучения протонами обнаружен ряд сигнальных путей, связанных с низкоэкспрессированными генами, таких как STAT5; сигнальный путь PD-1; отмечена MET-опосредованная активация сигнального пути PTK2, передача сигналов PDGF; CD22-опосредованная регуляция BCR; активация MAPK, опосредованная FCERI. Кроме вышеназванных сигнальных путей обращает на себя внимание активация процесса распада коллагена, FCGR3A-опосредованного фагоцитоза и FCGR3A-опосредованного синтеза интерлейкина-10 (IL10). При анализе обогащения среди высокоэкспрессируемых генов в ткани ПРГШ после протонного облучения были активированы процессы ороговения и биологического окисления.

**Заключение.** Облучение протонами при ПРГШ приводит к гиперэкспрессии генов, вовлеченных в регуляцию процессов ороговения и биологического окисления; гипоэкспрессии генов, связанных с подавлением сигнальных путей: STAT5, PD-1, MET-опосредованной активации сигнального пути PTK2, передачи сигналов PDGF; CD22-опосредованной регуляции BCR; активации MAPK, опосредованной FCERI, процесса распада коллагена, активации FCGR3A-опосредованного фагоцитоза и FCGR3A-опосредованного синтеза IL10. Все сигнальные пути гипоэкспрессированных генов функционируют в клетках ПРГШ, если негативного влияния на опухоль не оказывается извне (облучение или поступление противоопухолевых препаратов). Преобладание подавленных сигнальных путей над активированными, вероятнее всего, свидетельствует о снижении функционального потенциала клеток после облучения протонами. Дозозависимость эффектов ПТ обусловливает необходимость дальнейшего изучения изменений клеточных и молекулярно-генетических сигнатур ПРГШ после протонного облучения разными дозами.



**Ключевые слова:** плоскоклеточный рак головы и шеи, транскриптом, протонное облучение, сигнальные пути

**Конфликт интересов.** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

**Источник финансирования.** Работа выполнена при финансовой поддержке Министерства науки и высшего образования Российской Федерации (соглашение № 075-15-2021-1356 от 7.10.2021 (идентификатор РФ 0951.61321X0012, № 15.СИН.21.0011)). Обсчеты выполнены при финансовой поддержке Министерства науки и высшего образования Российской Федерации (соглашение № 075-15-2022-294 от 15.04.2022).

**Соответствие принципам этики.** Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено независимым комитетом по этике (протокол № 634 от 17.11.2021, протокол № 684 от 02.03.2022).

**Для цитирования:** Джуманиязова Э.Д., Вишнякова П.А., Чиркова М.В., Карпулевич Е.А., Еремина И.З., Гордон К.Б., Каприн А.Д., Фатхудинов Т.Х. Исследование транскриптома плоскоклеточного рака головы и шеи после протонного облучения. *Бюллетень сибирской медицины.* 2024;23(1):37–47. https://doi.org/10.20538/1682-0363-2024-1-37-47.

## INTRODUCTION

Head and neck squamous cell cancer (HNSCC) ranks 7th in the overall incidence of malignant neoplasms with 0.7 million new cases per year [1, 2]. Characteristic features of HNSCC are frequent recurrences and low 5-year survival rates for both localized and advanced stages of the disease (69 and 34%, respectively) [1]. Low patient survival is associated with late diagnosis, poor response to various treatments, and high recurrence rates [2–4]. In most cases, HNSCC is diagnosed at a locally advanced stage, for which radiation therapy (RT) with or without concomitant radiosensitizing chemotherapy is one of the main treatment approaches used in 80% of cases [5].

Proton therapy (PT) is one of the most promising types of corpuscular radiation. Its implementation into clinical practice allows to minimize the occurrence of radiation-related adverse events. The therapeutic effect of PT consists in persistent damage to the genetic material of tumor cells, leading to their death [6]. At the same time, the cytotoxic effect of protons is caused both by direct damage to the DNA chain of tumor cells and by indirect induction of reactive oxygen species (ROS) formation [7] and stimulation of apoptosis (due to caspase-3 activation by protons) [8].

In our previous review [9], we described the biological effects of PT. Nasopharyngeal squamous cell cancer is considered to be one of the main indications for PT due to its complex anatomy and proximity to critical anatomical structures and organs, such as optic chiasm, temporal lobes and brain stem, pharyngeal constrictor muscles, and salivary glands [10]. Several studies demonstrated a significant reduction in the incidence of acute post-radiation complications in patients with nasopharyngeal squamous cell carcinoma receiving PT compared to patients receiving classical RT [11].

The accumulating clinical experience in applying proton radiation therapy significantly outpaces basic radiobiological research and contributes to the increasing number of proton centers in different countries of the world [12]. However, limited knowledge about the molecular and genetic changes induced by PT in tumor cells hinders the development of new therapeutic and combination strategies. This study is devoted to the description of transcriptional changes in HNSCC cells after PT.

The aim of the study was to evaluate changes in the transcriptome of HNSCC tissue cells in patients after PT.

## MATERIALS AND METHODS

Biopsy material of tumor tissue was obtained from 3 patients with HNSCC before and after PT at the total irradiation dose of 10 isoGy (relative biological effectiveness (RBE) coefficient 1.1). All patients signed an informed consent to participate in the study. The study was approved by the independent Ethics Committee (Protocol No. 634 of 17.11.2021, Protocol No. 684 of 02.03.2022). The study was carried out in compliance with the ethical principles set out in the WMA Declaration of Helsinki "Ethical principles for medical research involving human subjects" amended in 2000 and Rules of Good Clinical Practice in the Russian Federation adopted by the order of the Ministry of Health of Russia No. 266 of 19.06.2003. The study participants were identified only by the patient number.

The patients were irradiated with protons using a fixed horizontal proton beam in the sitting position on the PT complex Prometheus (Protom, Russia). All patients underwent daily verification of the position using integrated cone beam computed tomography. The thickness of the slices was 1 mm. Patient fixation was performed using a reinforced thermoplastic mask and head restraints.

Biomaterial processing was performed using aseptic techniques and sterile materials. Total RNA was isolated from the tissue after homogenization with Teflon beads in QIAzol (Qiagen), followed by purification and concentration with the RNA Clean & Concentrator kit (Zymo Research). Quantitation was assessed using the Qubit 2.0 instrument (Invitrogen, Life Technologies). After isolation of 1 μg total RNA for sequencing, libraries were prepared on the Illumina platform using the TruSeq RNA Sample Prep Kit v2 with a 10-cycle enrichment step according to the manufacturer's recommendations.

The quality of RNA and the resulting libraries was checked using the Agilent 2100 Bioanalyzer system (Agilent Tec. Inc., USA). The RIN parameter for RNA was at least 7. The concentration of libraries was assessed by real-time PCR on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Final libraries were pooled in equimolar ratios before sequencing on the Illumina HiSeq 2500 platform using 50 base-pair paired-end reads. The Q20 parameter for all samples was > 97%, and the number of reads averaged 60.2 million per sample. Raw reads were processed using the RTA 1.17.21.3 and Casava 1.8.2 (Illumina).

The nf-core/rnaseq pipeline version 3.0 was used to obtain expression matrices from FASTQ files. The pipeline was run with the GRCh38 reference genome, alignment was performed using the STAR tool, and quantification was performed using the Salmon tool. The differential gene expression analysis was performed between tumor samples before and after PT. It was performed independently using several tools, such as DESeq2, EBSeq, limma-voom, NOISeq, and edgeR. For each tool, tables with differential gene expression scores were generated. The results obtained were compared using the Hobotnica metric [13].

Hobotnica is a tool for assessing the quality of differential expression tools. The tool is based on the quality quantification approach based on the ability to separate data from different experiments based on distance matrices. Cut-off values for differential gene expression were |log2FC| > 1, $p < 0.05$ for DESeq2, limma-voom and edgeR, $q > 0.9$ for NOISeq, and |log2FC| > 1, PPDE > 0.95 for EBSeq. For multiple comparisons, the Bonferroni correction and the Wald maximin criterion (extreme pessimism criterion) were used to calculate the *p* value. Based on the comparison results, DESeq2 performed better when comparing tumor samples before and after PT. The enrichment analysis was performed using the PANTHER 17.0 software. The threshold for statistical significance for including a signaling pathway in the list of enriched pathways was $p < 0.05$ (Table 1).

Table 1

**The assessment of the differential gene expression tool quality using the Hobotnica tool**

| Tool | Hobotnica score |
|---|---|
| DESeq2 | 1 |
| EBSeq | 0.96 |
| edgeR | 0.5 |
| limma-voom | 0.5 |
| NOISeq | 0.67 |

To establish significance, the data were analyzed by the paired Student's *t*-test using the GraphPad Prism 8 statistical software package (GraphPad Software). The significance level was set at $p \leq 0.05$. For multiple comparisons, the Bonferroni correction and the Wald maximin criterion (the criterion of extreme pessimism) were used, which are generally considered as the most cautious ones.

## RESULTS

The transcriptome analysis of HNSCC tumor tissue after proton radiation therapy (5 x 2 isoGy) at a total dose of 10 isoGy revealed 1,414 significantly differentially expressed genes. We identified 10 genes based on the minimum (with the most reduced expression) and maximum (with the most increased expression) LOG2FC values. The lowest expression after PT was observed in the following genes: *CLEC4E, IGHV2-70, P2RX1, SLC5A3, MYBPC1, RP11-551L14,1, FCRLA, FAM30A, IGHV2-26, IGHV2-5* (Table 2).

Table 2

**The most underexpressed genes in HNSCC biopsy samples after proton therapy**

| Genes | *p*-value | LOG2FC | Deciphering the name | Functions |
|---|---|---|---|---|
| *CLEC4E* | 1.89E-06 | -5.0 | C-Type Lectin Domain Family 4 Member E | Encodes a member of the C-type lectin / C-type lectin-like domain (CTL/CTLD) superfamily involved in cell adhesion, cell-to-cell signaling, glycoprotein metabolism, inflammation, and immune response |
| *IGHV2-70* | 8.57E-06 | -5.1 | Immunoglobulin Heavy Variable 2-70 | Provides antigen-binding activity and immunoglobulin receptor-binding activity; participates in the activation of the immune response, defense against another organism and phagocytosis |
| *P2RX1* | 3.99E-05 | -5.2 | Purinergic Receptor P2X 1 | The protein encoded by this gene belongs to the P2X family of G-protein-coupled receptors. It functions as an ATP-controlled ion channel and provides fast and selective permeability for cations |
| *SLC5A3* | 2.37E-05 | -5.2 | Solute Carrier Family 5 Member 3 | Involved in inositol metabolism; transmembrane transport of monosaccharides; import of myo-inositol across the plasma membrane |
| *MYBPC1* | 0.000185 | -5.2 | Myosin Binding Protein C1 | Involved in the contraction of transverse striated muscles |
| *RP11-551L14,1* | 0.011218 | -5.3 | No data | No data |
| *FCRLA* | 0.000857 | -5.3 | Fc Receptor Like Protein A | Involved in humoral immunity: antibody-induced destruction of IgG-coated antigens and cells |
| *FAM30A* | 0.000853 | -5.3 | Family With Sequence Similarity 30 Member A | Its exact function is unclear, but its activation has been linked to cancer |

Table 2 (continued)

| Genes | *p*-value | LOG2FC | Deciphering the name | Functions |
|---|---|---|---|---|
| *IGHV2-26* | 0.002372 | -5.4 | Immunoglobulin Heavy Variable 2-26 | Involved in activation of the immune response; defense against another organism; phagocytosis. |
| *IGHV2-5* | 0.018504 | -5.4 | Immunoglobulin Heavy Variable 2-5 | Provides antigen-binding activity and immunoglobulin receptor-binding activity. Activates immune response; defense against another organism; phagocytosis. |

The highest gene expression in HNSCC biopsy samples after PT was observed in the following genes: *PIK3R2; CTD-3074O7,11; GOLGA6L9; GP1BB; NPIPA2; RP11-96O20,4; AC008132,13; SNX31; RP1-127D3,4; RPL21P119* (Table 3).

In order to determine whether significantly highly expressed and low-expression genes belong to different signaling pathways, we performed the enrichment analysis for each of these groups of genes separately. In tumor tissue samples after PT, compared to tumor tissue before it, among low-expression genes, the signaling pathways presented in Table 4 were detected more than others. A number of signaling pathways related to low-expression genes were detected in HNSCC after PT, such as: STAT5; PD-1 signaling pathway; marked MET-mediated activation of PTK2 signaling pathway, PDGF signaling; CD22-mediated regulation of BCR; FCERI-mediated MAPK activation. In addition to the above signaling pathways, collagen degradation, FCGR3A-mediated phagocytosis activation, and FCGR3A-mediated interleukin (IL)-10 synthesis attract attention (Table 4).

In the enrichment analysis among highly expressed genes, the processes of keratinization and biological oxidation were activated in HNSCC tissue after PT (Table 5).

Table 3

**The most highly expressed genes in the samples of HNSCC biopsies after proton therapy**

| Genes | *p*-value | LOG2FC | Deciphering the name | Functions |
|---|---|---|---|---|
| *PIK3R2* | 0.001165114 | 4.9 | Phosphoinositide-3-Kinase Regulatory Subunit 2 | Phosphorylates phosphatidylinositol and similar compounds, creating secondary messengers important in growth factor signaling pathways |
| *CTD-3074O7,11* | 2.72E-06 | 4.6 | No data | Involved in the development of the eyes, limbs, heart, and reproductive system |
| *GOLGA6L9* | 1.59E-07 | 4.5 | Golgin A6 Family Like 9 | No data |
| *GP1BB* | 4.01E-10 | 4.3 | Glycoprotein Ib Platelet Subunit Beta | Promotes platelet adhesion. |
| *NPIPA2* | 8.65E-10 | 4.0 | Nuclear Pore Complex Interacting Protein Family Member A2 | Involved in mRNA transport and protein transport |
| *RP11-96O20,4* | 3.93E-11 | 3.8 | No data | No data |
| *AC008132,13* | 1.07E-11 | 3.6 | No data | No data |
| *SNX31* | 7.48E-13 | 3.4 | Sorting Nexin 31 | Involved in intracellular protein transport |
| *RP1-127D3,4* | 7.48E-13 | 3.4 | No data | No data |
| *RPL21P119* | 4.15E-12 | 3.4 | Ribosomal Protein L21 Pseudogene 119 | Pseudogene |

Table 4

**Signaling pathways of HNSCC associated with low-expression genes after proton therapy**

| Reactome pathways | Number of genes from the reference list of the database in a normal human population | Number of genes related to this signaling pathway in the submitted samples | Fold enrichment | *p* value | FDR Probability of false positives |
|---|---|---|---|---|---|
| Activation of STAT5 R-HSA9702518.2 | 10 | 4 | 15.63 | 3.15E-04 | 1.60E-02 |

Table 4 (continued)

| Reactome pathways | Number of genes from the reference list of the database in a normal human population | Number of genes related to this signaling pathway in the submitted samples | Fold enrichment | *p* value | FDR Probability of false positives |
|---|---|---|---|---|---|
| PD-1 signaling pathway R-HSA-389948.3 | 29 | 7 | 9.43 | 2.58E-05 | 2.22E-03 |
| Collagen degradation R-HSA-1442490.4 | 64 | 14 | 8.55 | 7.25E-09 | 6.03E-06 |
| MET -mediated activation of signaling pathway PTK2 R-HSA-8874081.2 | 30 | 6 | 7.81 | 2.42E-04 | 1.31E-02 |
| PDGF signaling R-HSA-186797.5 | 54 | 8 | 5.79 | 1.48E-04 | 9.44E-03 |
| AKT1 E17K signaling pathway in cancer R-HAS-5674400.2 | 26 | 5 | 7.51 | 9.44E-04 | 4.13E-02 |
| CD22-mediated BCR regulation R-HSA-5690714.3 | 67 | 9 | 5.25 | 1.13E-04 | 7.43E-03 |
| Interferon-gamma signaling R-HSA-877300.6 | 91 | 12 | 5.15 | 1.00E-05 | 1.09E-03 |
| FCERI-mediated MAPK activation R-HSA-2871796.3 | 89 | 11 | 4.83 | 4.03E-05 | 3.14E-03 |
| FCGR3A-mediated synthesis of IL-10 R-HSA-9664323.2 | 100 | 12 | 4.69 | 2.37E-05 | 2.11E-03 |

Here and in Table 5: 1 Fold enrichment is defined as the percentage of genes in the submitted samples belonging to the indicated pathway compared to the background set of genes. 2 +/-. A positive sign indicates overrepresentation of this category of genes in the experiment (more genes than expected). Conversely, a negative sign indicates underrepresentation. All signaling pathways showed overrepresentation.

Table 5

**Activated signaling pathways of HNSCC associated with highly expressed genes after proton therapy**

| Reactome pathways | Number of genes from the reference list of the database in a normal human population | Number of genes related to this signaling pathway in the submitted samples | Fold enrichment | *p* value | FDR Probability of false positives |
|---|---|---|---|---|---|
| Formation of the keratinizing membrane R-HSA-6809371.5 | 129 | 14 | 6.96 | 4.57E-08 | 1.14E-04 |
| Biological oxidation R-HSA-211859.3 | 220 | 15 | 4.37 | 3.83E-06 | 4.77E-03 |

The data obtained in the course of this research work are unique in their kind, as there is limited information on PT-induced changes at the transcriptome level in the literature. This is due, firstly, to the difficulty of collecting biopsies from patients, since for such analysis it is necessary to collect material before PT and after irradiation in order to identify significantly altered signatures. Secondly, not all centers have the necessary expensive equipment for PT, and often clinicians limit themselves to prescribing classical photon radiation therapy. Thirdly, transcriptional analysis and bioinformatic data processing are rather complicated and require highly qualified specialists.

## DISCUSSION

The transcriptome is a dynamically changing system influenced by various factors. As a precision oncology assay, the transcriptional analysis has only recently begun to be used [14, 15]. There are no data in the literature describing changes at the level of the HNSCC transcriptome induced by PT. Understanding the transcriptional heterogeneity of HNSCC contributes to the development of diagnostic and prognostic

biomarkers that will enable to select personalized therapy, leading to increased positive responses to antitumor therapy and improved outcomes / increased number of positive responses to treatment.

The key role for the activation of signal transducers and activators of transcription 5 (STAT5) has been found in many malignancies. In most cases, STAT5 enhances squamous epithelial cell growth, increases migration and invasion of cells in squamous cell cancer, and induces phenotypic and molecular changes associated with epithelial – mesenchymal transition [16]. In HNSCC, STAT5 activation correlates with enhanced tumor growth, invasion, and epithelial – mesenchymal transition [17]. Suppression of STAT5 was observed after PT in HNSCC.

Non-receptor protein – tyrosine kinase 2 (PTK2), also known as focal adhesion kinase (FAK), is a multifunctional regulator of cell signaling between tumor cells and the tumor microenvironment. Activated PTK2 is involved in the regulation of several cellular functions: adhesion, proliferation, and migration [18, 19]. Proteomic analysis showed that PTK2 / FAK overexpression is a biomarker of radioresistance of HNSCC. Combinations of PTK2 / FAK inhibition with radiation therapy are currently being investigated as a therapeutic strategy to improve local control in HPV-negative PRGS [20].

Activation of the PD-1 / PD-L1 signaling pathway is associated with the induction and maintenance of immune tolerance in tumor tissue by suppressing effector T cell functions [21]. PD-L1 expression levels in HNSCC correlate significantly with marked clinical progression and poor patient survival [22]. The activity of PD-1 and its ligands PD-L1 or PD-L2 is responsible for the activation, proliferation, and cytotoxic secretion of T lymphocytes [23]. Our transcriptome analysis revealed suppression of this signaling pathway.

IL-10 synthesis in HNSCC tumor tissue was also reduced after PT. In the process of carcinogenesis, IL-10 functions both as a pro-oncogenic cytokine, inhibiting antitumor immunity, and as an antitumor cytokine, performing an antianginal role [24]. Importantly, it is involved in the control of tumor cell proliferation and invasion via the JAK / STAT signaling pathway [25,26].

The PDGF / PDGFR signaling pathway plays a key role in tumor progression [27]. PDGF overexpression promotes tumor cell growth [28] and induces angiogenesis [29] by affecting cells in the tumor microenvironment, thereby inducing tumor progression. In addition, there is evidence that increased PDGF activity in the tumor is associated with drug treatment resistance caused by impaired capillary blood flow in the tumor due to increased interstitial fluid pressure [30]. In the present study, PT caused suppression of the PDGF signaling pathway in HNSCC.

The mitogen-activated protein kinase (MAPK) signaling pathway is a key mediator that integrates extracellular signals to control cell proliferation, survival, differentiation, senescence, and drug resistance [31]. HNSCC patients with high intratumoral expression of p-MAPK1/3 (p-ERK1/2) are characterized by worse survival [32]. It is believed that the altered kinase signaling network including EGFR, PDGFR, PAK1, PTK2 (FAK), and MAP2K2 seems to regulate aberrant changes in tumor tissue accompanying relapse [33].

In addition to the above, detection of changes in HNSCC after PT at the transcriptomic level will allow to divide patients into groups with a different prognosis and response to treatment. For example, the survival of patients with HNSCC can be stratified by the intensity of the keratinization process, especially in patients with non-HPV-associated HNSCC. Activation of the keratinization process in HNSCC is associated with a poor prognosis and shorter overall survival of patients [34].

## CONCLUSION

PT in HNSCC leads to overexpression of genes involved in the regulation of keratinization and biological oxidation processes as well as to underexpression of genes associated with suppression of signaling pathways: STAT5, PD-1, MET-mediated activation of PTK2 signaling pathway, PDGF signaling; CD22-mediated regulation of BCR; FCERI-mediated MAPK activation, collagen degradation, FCGR3A-mediated phagocytosis activation, and FCGR3A-mediated IL10 synthesis.

Summarizing the results obtained during the transcriptome study of HNSCC after PT at a total dose of 10 isoGy, the predominant number of suppressed signaling pathways over the activated ones is of great interest. At the same time, some of the detected signaling pathways correspond to the data described in the literature, while some of the pathways altered during the study differ from the available data. All signaling pathways of underexpressed genes function in HNSCC cells if there is no negative influence on the tumor from outside (irradiation or delivery of antitumor drugs). The predominance of suppressed

signaling pathways over activated ones most likely indicates a decrease in the functional potential of cells after proton therapy. The dose-dependence of proton therapy effects requires further study of changes in cellular and molecular-genetic signatures of HNSCC after proton therapy at different doses.

## REFERENCES


1. Sung H., Ferlay J., Siegel R.L., Laversanne M., Soerjomataram I., Jemal A., Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a Cancer Journal for Clinicians*. 2021;71(3):209–249. DOI: 10.3322/caac.21660.
2. Bhat G.R., Hyole R.G., Li J. Head and neck cancer: Current challenges and future perspectives. *In Advances in Cancer Research.* 2021;152(1):67–102. DOI: 10.1016/bs.acr.2021.05.002.
3. Johnson D.E., Burtness B., Leemans C.R., Lui W.Y., Bauman J.E., Grandis J.R. Head and neck squamous cell carcinoma. *Nature Reviews Disease Primers.* 2020;6(1):92–112. DOI: 10.1038/s41572-020-00224-3.
4. Lozano R., Naghavi M., Foreman K., Lim S., Shibuya K., Aboyans V. et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012;380(9859):2095–2128. DOI: 10.1016/S0140-6736(12)61728-0.
5. Borras J.M., Barton M., Grau C., Corral J., Verhoeven R., Lemmens V. et al. The impact of cancer incidence and stage on optimal utilization of radiotherapy: Methodology of a population based analysis by the ESTRO-HERO project. *Radiotherapy and Oncology*. 2015;116(1):45–50. DOI: 10.1016/j.radonc.2015.04.021.
6. Vitti E.T., Parsons J.L. The radiobiological effects of proton beam therapy: Impact on DNA damage and repair. *Cancers*. 2019;11(7):1–15. DOI: 10.3390/cancers11070946.
7. Răileanu M., Straticiuc M., Iancu D.A., Andrei R.F. Proton irradiation induced reactive oxygen species promote morphological and functional changes in HepG2 cells. *Journal of Structural Biology.* 2022;214(4):1–11. DOI: 10.1016/j.jsb.2022.107919.
8. Alan Mitteer R., Wang Y., Shah J., Gordon S., Fager M., Butter P.P. et al. Proton beam radiation induces DNA damage and cell apoptosis in glioma stem cells through reactive oxygen species. *Scientific Reports*. 2015;5(1):1–12. DOI: 10.1038/srep13961.
9. Jumaniyazova E.D., Smyk D.I., Vishnyakova P.A., Fatkhudinov T.Kh., Gordon K.B. Photon-and proton-mediated biological effects: what has been learned? *Life.* 2022;3(1):30–46. DOI: 10.3390/life13010030.
10. Nuyts S., Bollen H., Ng S.P., Corry J., Eisbruch A., Mendenhall W.M. et al. Proton therapy for squamous cell carcinoma of the head and neck: early clinical experience and current challenges. *Cancers*. 2022;14(11):1–18. DOI: 10.3390/cancers14112587.
11. Holliday E.B., Garden A.S., Rosenthal D.I., Fuller C.D., Morrison W.H., Gunn G.B. et al. Proton therapy reduces treatment-related toxicities for patients with nasopharyngeal cancer: a case-match control study of intensity-modulated proton therapy and intensity-modulated photon therapy. *International Journal of Particle Therapy.* 2015;2(1):19–28. DOI: 10.14338/IJPT-15-00011.1.
12. Tian X., Liu K., Hou Y., Cheng J., Zhang J. The evolution of proton beam therapy: Current and future status. *Molecular and Clinical Oncology*. 2018;8(1):15–21. DOI: 10.3892/mco.2017.1499.
13. Stupnikov A., Sizykh A., Budkina A., Sizykh A., Budkina A., Favorov A. et al. Hobotnica: exploring molecular signature quality [version 2; peer review: 2 approved]. *F1000Research*. 2022:10:1260. DOI: 10.12688/f1000research.74846.2.
14. Rodon J., Soria J.C., Berger R., Miller W.H., Rubin E., Kugel A. et al. Genomic and transcriptomic profiling expands precision cancer medicine: the WINTHER trial. *Nature Medicine.* 2019;25(5):751–758. DOI: 10.1038/s41591-019-0424-4.
15. Worst B.C., van Tilburg C.M., Balasubramanian G.P., Fiesel P., Witt R., Freitag A. et al. S. Next-generation personalised medicine for high-risk paediatric cancer patients–The INFORM pilot study. *European Journal of Cancer.* 2016;65(1):91–101. DOI: 10.1016/j.ejca.2016.06.009.
16. Choi S., Myers J.N. Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. *Journal of Dental Research.* 2008;87(1):14–32. DOI: 10.1177/154405910808700104.
17. Koppikar P., Lui V.W.Y., Man D., Xi S., Chai R.L., Nelson E. et al. Constitutive activation of STAT5 contributes to tumor growth, epithelial-mesenchymal transition, and resistance to EGFR targeting. *Clinical Cancer Research.* 2008;14(23):7682–7690. DOI: 10.1158/1078-0432.CCR-08-1328.
18. Zhou J., Yi Q., Tang, L. The roles of nuclear focal adhesion kinase (FAK) on Cancer: a focused review. *Journal of Experimental & Clinical Cancer Research*. 2019;38(1):1–11. DOI: 10.1186/s13046-019-1265-1.
19. Zhang Z., Li J., Jiao S., Han G., Zhu J., Liu T. Functional and clinical characteristics of focal adhesion kinases in cancer progression. *Frontiers in Cell and Developmental Biology*. 2022;10(1):1040311–1040335. DOI: 10.3389/fcell.2022.1040311.
20. Skinner H.D., Giri U., Yang L., Woo S.H., Story M.D., Pickering C.R. et al. Proteomic profiling identifies PTK2/FAK as a driver of radioresistance in HPV-negative head and neck cancer. *Clinical Cancer Research*. 2016;22(18):4643–4650. DOI: 10.1158/1078-0432.CCR-15-2785.
21. Chen L., Han X. Anti–PD-1/PD-L1 therapy of human cancer: past, present, and future. *The Journal of Clinical Investigation.* 2015;125(9):3384–3391. DOI: 10.1172/JCI80011.

22. Cui P., Jing P., Liu X., Xu W. Prognostic significance of PD-L1 expression and its tumor-intrinsic functions in hypopharyngeal squamous cell carcinoma. *Cancer Management and Research.* 2020;1:5893–5902. DOI: 10.2147/CMAR.S257299.
23. Han Y., Liu D., Li L. PD-1/PD-L1 pathway: current researches in cancer. *American Journal of Cancer Research*. 2020;10(3):727–742.
24. Howell W.M., Rose-Zerilli M.J. Cytokine gene polymorphisms, cancer susceptibility, and prognosis. *The Journal of Nutrition*. 2007;137(1):194S–199S. DOI: 10.1093/jn/137.1.194S.
25. Béguelin W., Sawh S., Chambwe N., Chan F.C., Jiang Y. IL10 receptor is a novel therapeutic target in DLBCLs. *Leukemia.* 2015;29(8):1684–1694. DOI: 10.1038/leu.2015.57.
26. Han Y., Ding Z., Chen B., Liu Y., Liu Y. A novel inflammatory response–related gene signature improves high-risk survival prediction in patients with head and neck squamous cell carcinoma. *Frontiers in Genetics.* 2022;13(1):1–15. DOI: 10.3389/fgene.2022.767166.
27. Heldin C.H. Targeting the PDGF signaling pathway in tumor treatment. *Cell Communication and Signaling.* 2013;11:1–18. DOI: 10.1186/1478-811X-11-97.
28. Pietras K., Sjöblom T., Rubin K., Heldin C.H., Östman A. PDGF receptors as cancer drug targets. *Cancer Cell*. 2003;3(5):439–443. DOI: 10.1016/s1535-6108(03)00089-8.
29. Lindahl P., Johansson B.R., Leveen P., Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science.* 1997;277(5323):242–245. DOI: 10.1126/science.277.5323.242.
30. Heldin C.H., Rubin K., Pietras K., Östman A. High interstitial fluid pressure—an obstacle in cancer therapy. *Nature Reviews Cancer.* 2004;4(10):806–813. DOI: 10.1038/nrc1456.
31. Braicu C., Buse M., Busuioc C., Drula R., Gulei D., Raduly L. et al. A comprehensive review on MAPK: a promising therapeutic target in cancer. *Cancers.* 2019;11(10):1–25. DOI: 10.3390/cancers11101618.
32. Theocharis S., Kotta-Loizou I., Klijanienko J., Giaginis C., Alexandrou P., Rodriguez J. et al. Extracellular signal-regulated kinase (ERK) expression and activation in mobile tongue squamous cell carcinoma: associations with clinicopathological parameters and patients survival. *Tumor Biology*. 2014;35:6455–6465. DOI: 10.1007/s13277-014-1853-9.
33. Kaneko T., Zeng P.Y., Liu X., Abdo R., Barrett J.W., Zhang Q. et al. Proteome and phosphoproteome signatures of recurrence for HPV+ head and neck squamous cell carcinoma. *Communications Medicine.* 2022;2(1):95–110. DOI: 10.1038/s43856-022-00159-8.
34. Cooper T., Biron V.L., Adam B., Klimowicz A.C., Puttagunta L., Seikaly H. Association of keratinization with 5-year disease-specific survival in oropharyngeal squamous cell carcinoma. *JAMA Otolaryngol Head Neck Surg.* 2015;141(3):250–256. DOI: 10.1001/jamaoto.2014.3335.

## Authors' contribution

Jumaniyazova E.D. – analysis and interpretation of the data, drafting of the manuscript. Vishnyakova P.A. – conception and design, critical revision of the manuscript for important intellectual content. Chirkova M.V., Karpulevich E.A. – bioinformatics analysis of the results obtained. Eremina I.Z., Kaprin A.D. – final approval of the manuscript for publication. Gordon K.B. – delivery of the biological material, critical revision of the manuscript for important intellectual content. Fatkhudinov T.H. – conception and design, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication.

## Authors' information

**Jumaniyazova Enar D.** – Post-Graduate Student, Teaching Assistant, Department of Histology, Cytology and Embryology, RUDN Medical Institute; Research Intern, Laboratory for Molecular Pathophysiology of the Cell, RUDN Research Institute of MCM, Moscow, enar2017@yandex.ru, http://orcid.org/0000-0002-8226-0433

**Vishnyakova Polina A.** – Cand. Sci. (Biology), Associate Professor, Department of Histology, Cytology and Embryology, RUDN Medical Institute; Head of the Laboratory for Regenerative Medicine, V.I. Kulakov National Medical Research Center of Obstetrics, Gynecology and Perinatology; Head of the Laboratory for Molecular Pathophysiology of the Cell, RUDN Research Institute of MCM, Moscow, p_vishnyakova@oparina4.ru, http://orcid.org/0000-0001-8650-8240

**Chirkova Miroslava V.** – Laboratory Assistant, ISP RAS, Moscow, mirachirkova@ispras.ru , http://orcid.org/0009-0008-3659-0360

**Karpulevich Evgeny A.** – Researcher, ISP RAS, Moscow, karpulevich@ispras.ru, http://orcid.org/0000-0002-6771-2163

**Eremina Irina Z.** – Cand. Sci. (Biology), Associate Professor, Department of Histology, Cytology and Embryology, RUDN Medical Institute, Moscow, eremina_iz@rudn.university, http://orcid.org/0000-0002-5093-6232

**Gordon Konstantin B.** – Cand. Sci. (Med.), Associate Professor, Department of Histology, Cytology and Embryology, RUDN Medical Institute, Radiotherapist, Researcher, Department of Proton and Photon Radiation Therapy, Tsyb Medical Radiological Research Center, Moscow, drgordonkb@gmail.com,: http://orcid.org/0000-0002-2759-297X

**Kaprin Andrey D.** – Dr. Sci. (Med.), Academician of the Russian Academy of Sciences, Head of the Department of Oncology and Radiology named after Academician Kharchenko, RUDN Medical Institute; Director General, NMRC Radiology; Moscow, kaprin-ad@rudn.ru, http://orcid.org/0000-0001-8784-8415

**Fatkhudinov Timur H.** – Dr. Sci. (Med.), Professor, Head of the Department of Histology, Cytology and Embryology, RUDN Medical Institute, Deputy Director for Research, RUDN Medical Institute; Deputy Director, A.P. Avtsyn Research Institute of Human Morphology; Director, RUDN Research Institute of MCM, Moscow, tfat@yandex.ru, http://orcid.org/0000-0002-6498-5764

(✉) **Jumaniyazova Enar D.,** enar2017@yandex.ru

Received 03.09.2023;
approved after peer review 27.10.2023;
accepted 16.11.2023