



Drug Tolerant Cells: An Emerging Target With Unique Transcriptomic Features

Divya Niveditha , Harshita Sharma, Anirudha Sahu, Syamantak Majumder, Rajdeep Chowdhury and Shibasish Chowdhury 

Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani, Pilani, India.

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ABSTRACT: Long-term outcome of cancer therapy is often severely perturbed by the acquisition of drug resistance. Recent evidence point toward the survival of a subpopulation of tumor cells under acute drug stress that over time can re-populate the tumor. These transiently existing, weakly proliferative, drug-tolerant cells facilitate tumor cell survival until more stable resistance mechanisms are acquired. From a therapeutic perspective, understanding the molecular features of the tolerant cells is critical to attenuation of resistance. In this article, we discuss the transcriptomic features of drug-tolerant osteosarcoma cells that survive a high dose of cisplatin shock. We present the unique transcriptome of the minimally dividing tolerant cells in comparison with the proliferative persisters or resistant cells derived from the tolerant cells. Targeting the tolerant cells can represent an efficient therapeutic strategy impeding tumor recurrence.

KEYWORDS: Osteosarcoma, RNA sequencing, drug resistance, drug-tolerant persister, epigenetic alteration

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CORRESPONDING AUTHORS: Rajdeep Chowdhury, Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani, Vidyavihar, Pilani 333031, Rajasthan, India. Email: rajdeep.chowdhury@pilani.bits-pilani.ac.in

Shibasish Chowdhury, Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani, Vidyavihar, Pilani 333031, Rajasthan, India. Email: shiba@pilani.bits-pilani.ac.in

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Acquired resistance to conventionally used drugs has now become a global concern and remains a key obstacle to successful cancer therapy. Substantial research pertaining to drug resistance carried out over the last decade has elucidated various mechanisms like drug efflux, drug metabolism, and engagement of alternative survival pathways as crucial strategies employed by tumor cells for manifesting drug resistance. In addition, heritable genetic alterations conferring tumor cells with a selective survival advantage have also been considered as a cause of drug resistance. In this regard, a string of recent studies has emphasized an interesting yet least explored aspect of the acquisition of drug resistance by tumor cells. It suggests that cells showing resistance to drugs do not necessarily require a stable heritable genetic alteration; instead, a switch to transitory, “drug-tolerant” phenotype emerging under acute drug stress holds the key to re-population of the tumor. This has immense implication from a clinical scenario as it might be necessary for understanding tumor recurrence in cancer patients. It is observed that following a toxic drug insult that kills a vast majority of tumor cells, a very small subpopulation of tumor cells eventually does survive; these drug-tolerant cells remain in a non-dividing or dormant state, only to re-establish the tumor following the withdrawal of drug pressure. These tolerant cells do not only take up the burden of maintaining the tumor cell population under acute drug stress, they also represent the pool of cells that eventually give rise to resistant tumor cells. Considering the clinical significance of drug-tolerant tumor cells, there has been a

recent drive to characterize the molecular dependency of these drug-tolerant tumor cells. Based on the current understanding, it is conceived that the “tolerant” cells evade drug pressure primarily through selective epigenetic alterations facilitating their survival, and this drug tolerance is reversed if the cells are subjected to a “drug holiday,” implicating the importance of putative epigenetic mechanisms favoring their survival.¹ Importantly, the expression of drug efflux pumps was not found to be elevated in the tolerant cells further emphasizing the significance of epigenetic alteration in the acquisition of drug-tolerant phenotype. For example, Sharma et al² for the first time reported that, the establishment of drug resistance in “non-small cell lung carcinoma” requires the epigenetic regulator histone demethylase *KDM5A* to maintain the chromatin state under acute drug pressure, and a knockdown of *KDM5A* is sufficient to reduce emergence of drug-tolerant persister population.² Dawson et al³ further reported a similar phenomenon in the microbial cells and termed it as “persistence,” where, in response to antibiotics, bacterial populations avoided extinction by adopting a subpopulation of drug-insensitive dormant cells.³ Considering both tumor cells and microbes, it is evident that “persistence” poses a major obstacle for treatment; however, its importance has been very much under-appreciated and has been relatively underexplored.

Therefore, to have more insights into the underlining mechanism(s) of drug tolerance, we analyzed and compared the genome-wide mRNA expression pattern of osteosarcoma



(OS) cells surviving a lethal drug dose.⁴ The overall transcriptomic comparison was carried out among four different groups of cells, namely, untreated parental OS cells (OS), non-dividing cells that survived a high drug shock (drug-tolerant persisters, OS-Ps), cells that resumed proliferation yielding the “extended persisters” after a drug stress (OS-EP), and drug-resistant cells derived by consecutive drug treatment followed by clonal selection of the surviving populations (OS-R). The objective of the study was to characterize the sequential transcriptomic alterations associated with the emergence of drug-tolerant cells and subsequent resistant cells in OS. Osteosarcoma cells were selected for the study because OS is an aggressive cancer and, despite the current dual treatment strategy of both chemotherapy and surgery, a vast majority of patients are unresponsive to the standard treatment.⁵⁻⁷ Importantly, the primary reason for treatment failure in OS is identified as drug resistance. Therefore, it is imperative to identify the molecular basis for therapy resistance and devise ways to target therapy resistance in OS. Cisplatin has been the mainstay in OS therapy for more than thirty years and hence cisplatin was chosen as the drug of choice for this study. While analyzing the acute response to cisplatin in OS cells, in corroboration to earlier reports related to drug tolerance,⁸⁻¹³ we constantly observed a small subpopulation of cells that maintained viability under conditions where the vast majority of cells were rapidly eradicated. These persisters were mostly non-dividing, but a small percentage of them eventually resumed proliferation yielding the “extended persisters” which re-established the OS cell population again. We assumed that these transient drug-tolerant OS cells represent the “rebels” that withstand an onslaught of drug pressure and take the “onus” of protecting the OS tumor population. Exploring the transcriptome of such cells has significant potential therapeutic implications in the development of therapies directed at the biology of robust cells, supporting tumor survival and promoting drug resistance.

Distinct differences were observed when we compared the transcriptomic profiles between each group of cells. Probably, the groups with the most unique transcriptomic pattern were the OS-Ps, which survived the acute drug shock. Interestingly, there was a drastic increase in the number of transcripts down-regulated in OS-P cells compared with parental OS control; however, they still survived the drug shock. In contrary, the transcriptomic pattern of OS-R, although distinctively different from OS-P, showed relatively less drastic differences from the parental OS cells. We assume that a significant drift in the transcriptomic pattern in the OS-Ps is probably attributed to the extreme onslaught of drug pressure; however, as the cells, by virtue of their unique transcriptomic expression pattern, cope with it, the radical divergence in expression profiles minimizes. Interestingly, the resistant OS-R cells could still be sensitized to cisplatin if given a “drug holiday.” This hints toward a probable epigenetic shift responsible for the “tolerant” state and their subsequent acquisition of resistance, rather than a stable,

Table 1. Expression status of multidrug transporters belonging to ABCC gene family in cisplatin shock (OS-P) cells.

GENE NAME	LOG2 (FOLD_CHANGE)
<i>ABCC1</i>	0.92
<i>ABCC2</i>	0.66
<i>ABCC4</i>	-3.66
<i>ABCC4</i>	-1.55
<i>ABCC4</i>	0.95
<i>ABCC5</i>	-3.44
<i>ABCC5</i>	-0.06
<i>ABCC5</i>	1.15
<i>ABCC6</i>	-
<i>ABCC9</i>	-1.86
<i>ABCC9</i>	0.35
<i>ABCC10</i>	0.98

Abbreviations: OS, osteosarcoma; OS-P, drug-tolerant persisters.

acquired, heritable, genetic mutation. We further observed that the tolerant cells (OS-P) were in a non-dividing state for a period of time; in corroboration to the above, the genes associated with cell proliferative pathways such as Wnt signaling, mammalian target of rapamycin (mTOR) signaling, and Ras-MAPK (mitogen-activated protein kinase) signaling were significantly down-regulated in OS-P cells. We presume that because the cells in the “persister” phase channel most of their resources to adapt and survive the acute drug stress, suppression of pathways involved in proliferation is quite logical. In addition, an increased number of transcription factors were found to be down-regulated in OS-P cells, suggesting a possible transcriptional shutdown post drug shock; this included master transcription factors like *HIF-1 α* (a subunit of transcription factor produced in response to hypoxia stress). This might be an optimization strategy adapted by the OS-P cells to evade extinction under the acute onslaught of drug pressure. An important indication toward a probable non-genetic factor-dependent acquisition of tolerance came from the expression pattern of drug transporters and resistance-associated genes. The expression of most multidrug transporters such as the ABCC family of genes and others that have an established function in the export of drugs, thus minimizing toxicity associated with the drugs, showed either baseline or reduced expression (Table 1). Similarly, a vast majority of genes implicated in resistance such as *MSH2*, *BIRC2*, *PTEN*, *COL11A1*, *MAPK8*, *MDM2*, *GCLM*, *MMP7*, *TPM1*, and *RECQL* were down-regulated along with *HIF-1 α* , and genes like *SMARCA4* (involved in chromatin remodeling) was up-regulated, hinting toward mechanisms beyond drug efflux to be involved in drug

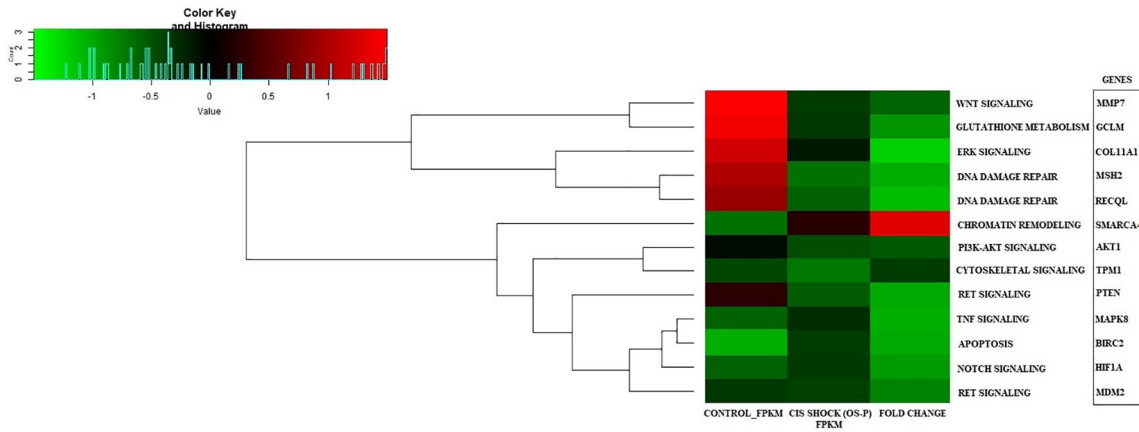


Figure 1. Heatmap representing expression pattern of resistance-associated genes in cisplatin shock (OS-P) cells. The probable pathways that these genes can directly or indirectly regulate, as analyzed through KEGG database, is also indicated. KEGG indicates Kyoto Encyclopedia of Genes and Genomes; OS, osteosarcoma; OS-P, drug-tolerant persisters.

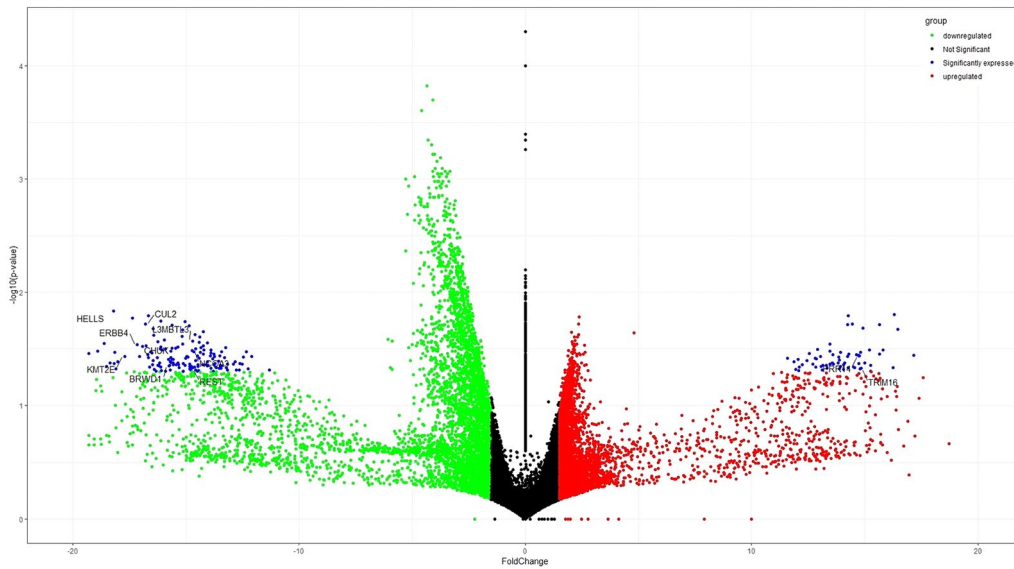


Figure 2. Volcano plot representing differential expression pattern of transcripts in OS-P cells compared with OS, along with labeled epigenetic genes. The up-regulated transcripts are in red and down-regulated transcripts are in green. Transcripts with fold change “±10” are in blue. Epigenetic genes having more or less than 10-fold differential expression are labeled. OS indicates osteosarcoma; OS-P, drug-tolerant persisters.

tolerance of the OS-P cells (Figure 1). This molecular evidence persuaded us to analyze the expression pattern of transcripts associated with epigenetic alterations in the tolerant OS-P cells. Indeed, an analysis of transcripts involved in epigenetic control showed drastic alterations. A list of genes with epigenetic functions showing differential expression in OS-P in comparison with OS cells is listed in Figure 2. Among the epigenetic modulators, noteworthy was the altered expression pattern of selected genes involved in chromatin organization, like histone de-acetylases (HDACs), SIRT, and specific Jumonji-C-demethylases (KDMs). The involvement of KDMs with drug tolerance has been previously reported.¹⁴⁻¹⁶ However, their precise role in imparting tolerance is required to be further explored in subsequent studies to unravel a potentially novel avenue of therapeutic strategy against tumor recurrence. Recent studies indicate that the emergence of cancer stem cells (CSCs), which

might re-populate a tumor after chemotherapy, can be induced as a result of a phenomenon called epithelial to mesenchymal transition (EMT). However, EMT in tumor cells not only causes enhanced metastasis or CSC development but is also known to contribute toward drug resistance. We were therefore interested to analyze the expression pattern of EMT specific genes in the drug-tolerant cells. Genes like *Vimentin* and axon guidance molecules such as *Semaphorins*, with roles in EMT and cancer cell invasion, showed enhanced expression in OS-P; however, it is subjected to further analysis whether EMT is fundamentally associated with drug tolerance. To have a holistic idea on the subset of transcripts that might be involved in imparting drug tolerance, we embarked on identifying key genes, that is, the differentially expressed genes that have overlapping functions in the following three domains, as per their Gene Ontology functional annotation: intracellular signaling,

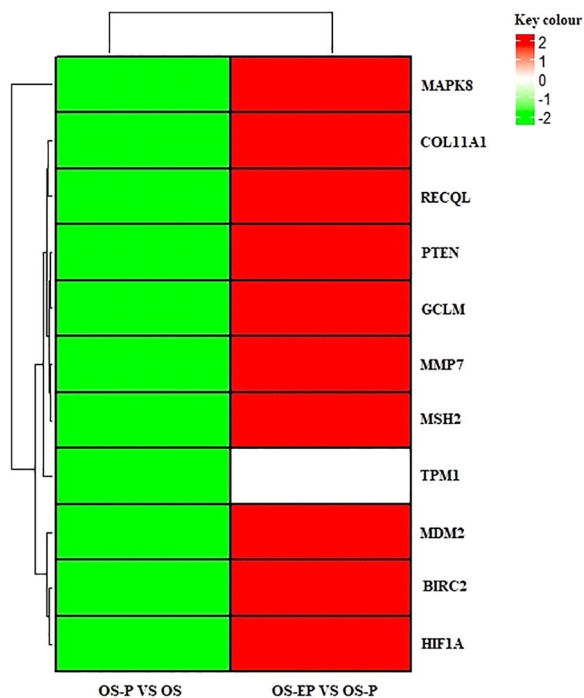


Figure 3. Heatmap showing the reversed expression pattern of resistance-associated genes in OS-P cells compared with OS-EP. OS indicates osteosarcoma; OS-EP, cells that resumed proliferation yielding the “extended persisters” after a drug stress; OS-P, drug-tolerant persisters.

receptor-mediated signaling, and functions regulating other diverse cellular mechanisms. The comparative transcriptome analysis between OS-P and OS cells revealed differential expression of 115 key genes, which included *CHUK*, *REST*, *SMARCA4*, *MAP3K7*, *CTCF*, and *HDAC2*, among others. We assume that in the drug-tolerant cells, the epigenetic factors might directly or indirectly regulate the key genes, which might be vital to OS-P cell survival under acute drug stress. However, further studies are required to understand the effect of the key genes identified. As mentioned earlier, immediately after drug exposure, the OS-P cells progressed into a non-proliferative phase in order for them to revive their population and proliferative activity. We hence also compared the transcriptomic profile between OS-P and OS-EP cells. Interestingly, the genes reportedly involved in resistance, which were down-regulated in OS-P (*Col11A1*, *HIF-1 α* , *MAPK8*, *BIRC2*, *GCLM*, *MDM2*, *RECQL*, *PTEN*, *TPM1*, and *MMP7*) were found to be up-regulated in OS-EP (Figure 3). This again portrays that the molecular phenotype of tolerance is different from cells that have survived the tolerant state and resumed proliferation. Hence, it is important to identify features of drug-tolerant cells to effectively uproot tumor resistance. Finally, a transcriptomic profile of the resistant cells (OS-R) that emerged from the OS-EP cells was compared with the parental OS cells. This analysis showed that the number of genes differentially regulated in OS-R was significantly less, compared with the comparisons between OS and OS-P or OS and OS-EP. We, therefore, believe that, as cells acquire

resistance, a selected set of genes are required to maintain the resistance phenotype. Gene ontology enrichment analysis identified a subset of key genes that included genes implicated in tumor pathogenesis such as PI3-Akt signaling and MAPK signaling, TGF- β (transforming growth factor beta) signaling, cAMP (cyclic adenosine monophosphate) signaling, and Ras signaling. Furthermore, through the transcriptomic comparisons between each set, we have also identified pathways that have been extensively de-regulated during the process of acquisition of resistance. This included pathways such as PI3-Akt, MAPK, and NF κ B signaling, which might have a critical role in drug tolerance and resistance acquisition.

Recent reports suggest that a tumor may harbor small subpopulations of cells that promote survival under drug pressure. These tolerant cells can survive strong apoptotic stimuli until stable long-term resistance is acquired. From the clinical perspective, it is therefore important to identify this subpopulation of tolerant tumor cells, understand their underlying mechanism of action, and develop effective strategies that can inhibit drug evasive molecular program adopted by the tolerant cells. In this context, our study for the first time provides comprehensive information on how drug treatment influences sequential global mRNA profile expression changes in OS cells leading to eventual attainment of drug resistance.⁴ Although several studies have reported transcriptome analysis in OS, none of them presents and compares transcriptomic alterations during the process of acquisition of resistance to the most widely used drug cisplatin.¹⁷⁻²² Overall, our study provides a molecular picture of non-dividing OS cells that can tolerate drug pressure, ultimately leading to the development of resistant phenotype. In each phase during the acquisition of resistance, the OS cell response systematically changed, as depicted by their transcriptome profiling. We have thus provided critical hints toward potential markers that can be targeted to prevent the emergence of tolerant cells and subsequent resistant population. Future studies, targeting the markers of drug tolerance could be designed in combination with conventional therapy to effectively eradicate OS, preventing recurrence.

Author Contributions

All authors contributed significantly towards analysis of the data contents and drafting of this manuscript.

ORCID iDs

Divya Niveditha  <https://orcid.org/0000-0003-0548-0042>
Shibashish Chowdhury  <https://orcid.org/0000-0001-9290-3802>

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