Integrative Transcriptomic Analysis of Two Cell Lines Elucidates the Architecture of Endoplasmic Reticulum Stress Signaling in Glioblastoma

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***Abstract*—The endoplasmic-reticulum (ER) stress response represents a pivotal cellular process, triggered by a variety of stimuli implying incorrect protein folding in the ER. Solid evidence implicates ER stress-induced dysfunction as major contributors in many cancers, among which brain cancers. In this work, an integrative analysis in two transcriptomic datasets scrutinizes the regulatory potency of ER stress mechanisms in glioblastoma. Besides confirming the results of the initial analyses, the integration of both datasets manages to study the effect of cellular stress in conjunction to the dysregulation of the IRE1α branch of the ER stress signaling pathways. Given the respective diversified phenotypic profile of glioblastomas, ranging from increased proliferation to enhanced migration and aggressiveness as a result of the IRE1α molecular switch, this study suggests potential target groups of master regulators of the ER stress pathways, through the application of an established, exhaustive computational framework.**

I. INTRODUCTION

THE tumour microenvironment, in particular, hypoxia and nutrient limitation, can lead to perturbations of Endoplasmic Reticulum (ER) functions, thereby resulting in the activation of an adaptive response, named the Unfolded Protein Response (UPR) [1], [2]. Eukaryotic cells have developed this evolutionarily conserved adaptive mechanism, in order to clear unfolded proteins and restore ER homeostasis[3]. In cases where ER stress cannot be reversed, cellular functions deteriorate, often leading to cell death. ER stress response (Fig.1) as a result of accumulation of unfolded protein recruits BiP to the ER lumen which dissociation from IRE1α, ATF6 and PERK leads to their activation. Upon dimerization and autophosphorylation, IRE1α splices XBP-1 mRNA, producing an active transcriptional factor XBP-1. IRE1α can also recruit TRAF2 and ASK1, leading to downstream activation of Bim and inhibition of Bcl-2 via JNK activation and CHOP activation through p38 MAPK phosphorylation. CHOP can induce transcriptional activation of genes that contribute to cell death, whereas PERK phosphorylates elF2α and attenuates protein translation with the exception of ATF4, which then induces expression of CHOP and GADD34. Activated ATF6 ultimately mediates expression of several components important for protein folding, degradation, and ER expansion. While representing in general, a pivotal cellular homeostatic mechanism, in the case of cancer, UPR primarily plies tumor cells with the ability to cope with stress and adapt for survival. In addition to its role in cellular adaptation, the UPR, and in particular IRE1α signalling, have been proposed to play significant roles during tumour development. This was supported by the identification of somatic mutations in the IRE1α gene [4] or the dysregulation of ER stress targets in various cancers [5-7]. Moreover, the RNAse activity of IRE1α and the XBP1 transcription factor, whose mRNA is spliced by the combined action of IRE1α RNAse activity and a yet unknown ligase, have also been found to be necessary for tumor formation and growth in multiple myeloma, glioblastoma and transformed embryonic fibroblast [8-10].

Although these elements demonstrate the prime role of IRE1α in tumorigenesis, the molecular mechanisms

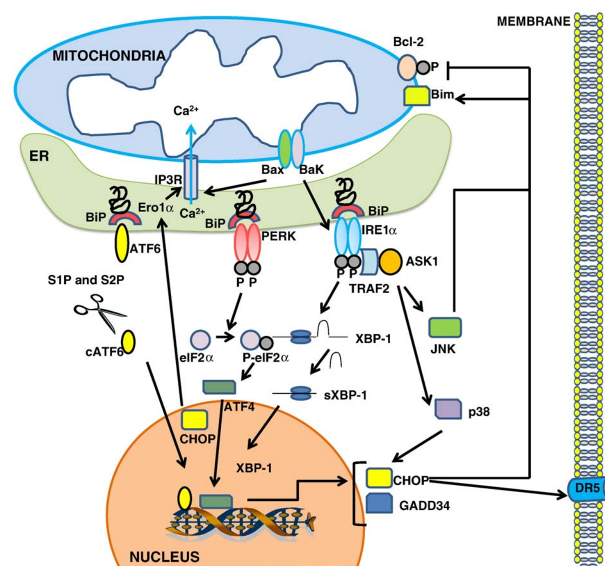

Fig. 1. Illustration of the Endoplasmic reticulum (ER) stress signaling (borrowed from [3]). The regulatory actions of the three sensors located at the ER membrane IRE1α, ATF6 and PERK are summarized.

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connecting this protein to cancer biology remain unclear. We and others have previously shown a role for IRE1α signalling in the regulation of a wide range of glioblastoma features including cell morphology, cytoskeleton organization, angiogenesis, tumors growth and cell invasion [11-13]. Using gene expression profiling we identified SPARC, a matrix protein and PER1, a component of the circadian clock, as IRE1α RNase activity direct degrading substrates, mediating IRE1α regulation of glioblastoma infiltration/invasion [12, 14]. Indeed, inhibition of IRE1α activity resulted in enhanced migration/invasion of glioblastoma cells both in vitro and in vivo through mechanisms depending on extracellular matrix remodeling and Rho GTPase signaling for SPARC and through the regulation of pro-inflammatory chemokines for PER1. In all cases, expression levels of these IRE1α substrates correlating with high IRE1α activity in patient samples also correlated with poor prognosis in patients, thereby pointing towards oncogenic functions for IRE1α. Moreover, the analysis of clinical samples (Fig. 2) revealed that low *PER1* mRNA expression and high *XBP1* mRNA splicing correlated with poorer prognoses [14]. Overall, these results identify IRE1α as a master regulator of cellular homeostasis in tumors, and provide the rationale for the development of IRE1α targeted therapies in cancer cells. These results have provided implications on glioblastoma progression for specific IRE1α substrates, so an integrated and global view of IRE1α-mediated functions in glioblastoma will aid the elucidation of the regulatory complexity of its progression.

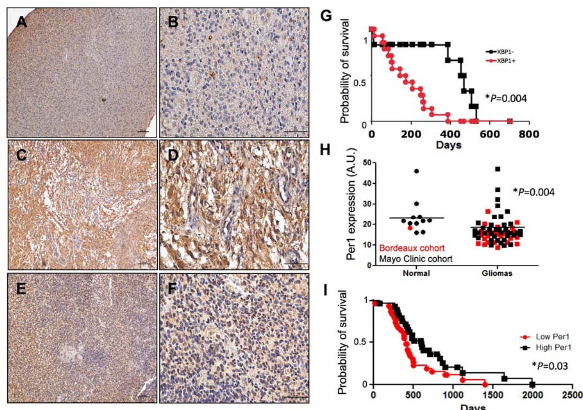

Fig. 2. Pathological relevance of IRE1 signaling in glioblastoma. (A-F) Immunohistochemical analysis of 3 typical glioblastoma sections using anti sXBP1 antibodies. Higher magnification of A, C and E are shown in B, D and F respectively. (G) Kaplan-Meier survival curves of patients displaying negative sXBP1 staining (6 ; XBP1-) or positive sXBP1 staining (14 ; XBP1+). P= 0.004; Log-rank test. (H) qPCR analysis of *PER1* mRNA expression in 60 glioblastoma cancer samples and 12 normal brain tissues. Bordeaux cohort is indicated in red, Mayo Clinic cohort in black. The results are expressed in arbitrary units as a ratio of *PER1* transcripts to *Rplp0* transcripts. The P value is indicated. (I) High (n=31) and low (n=29) *PER1* mRNA level correlates with patient survival. Values were plotted in Kaplan-Meier survival curves. Statistical difference between the 2 groups is indicated. Statistical difference between the two groups in indicated P=0.03; Log-rank test.

In this work the integration of two microarray datasets takes place, where the effect of the functionality of IRE1α signalling is monitored by contrasting gene expression in glioblastoma to that observed when IRE1α activity is impaired by a dominant negative clone. Given the respective diversified phenotypic spectrum of glioblastomas, encompassing from increased proliferation to enhanced migration and aggressiveness as a result of the IRE1α molecular switch, this study suggests potential target groups of master regulators of the ER stress pathways. This is done through the application of an established, exhaustive computational framework [15]. Goal is the elucidation of the intricate regulatory architecture of this extremely lethal cancer type thus pursuing the translation of these notions to innovative, more effective therapeutic approaches.

II. Materials and Methods

Glioma cells lines were utilized to monitor the impact at the transcriptomic level of the IRE1α ER stress response by comparing the case where the cells are stimulated with a ‘control ‘plasmid (EV cell line) to the dominant negative form of IRE1α protein (DN-IRE1α), both in basal and stress conditions. The integrative dataset comprises two datasets, which extraction processing is described in [11],[12]. Total RNA was extracted using Trizol (Invitrogen). RNA integrity was verified on an Agilent 2100 Bioanalyzer. For each of the samples, total RNA was reverse transcribed into cDNA, followed by in vitro transcription and biotin labeling to generate cRNA (Enzo Biochem, Farmingdale, NY, USA). The fragmented, biotin-labeled cRNA was hybridized to Human Genome U133 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA) containing approximately 22,000 probes. Pre-processing and normalization of the Affymetrix array signals was performed with the use of the Gene ARMADA software [16]. Data were normalized according to the GCRMA method, while variance was added to minimize the impact of spurious signals in the selection process. Statistical selection, (t-test for the comparison between the control categories and one-way ANOVA for the 4 categories comparison, threshold p-value 0.05 and FDR < 0.1), provided the significantly differentially expressed (DE) genes among the 4 categories (EV, EV_stress, DN, DN_stress).

The significant gene list was further subjected to dual hierarchical clustering (gene and sample clustering linkage method: Average, distances: Cosine, Euclidean, Pearson correlation) in Gene ARMADA. The optimal clustering tree (Pearson correlation distance) was selected according to the distance, which top-performed in clusteirng the samples together in their categories. Then genes sub-clusters were picked on the basis of their similarity in expression, either for prioritized statistical enrichment analysis exploiting the Gene Ontology through utilization of the StRAnGER tool (Hypergeometric score < 0.05, bootstrap score <0.1) [17], or analysis of their promoter regions exploiting the Genomatix ElDorado database and the MatInspector software [18]. The

parameters used were as follows: Library version: Matrix Library 8.0, Matrix group: Vertebrates, Transcription Factor sites common to: 70% of input sequences, Core similarity: 0.75, Matrix similarity: Optimized, and p-value cut-off was set at 10^{-20}.

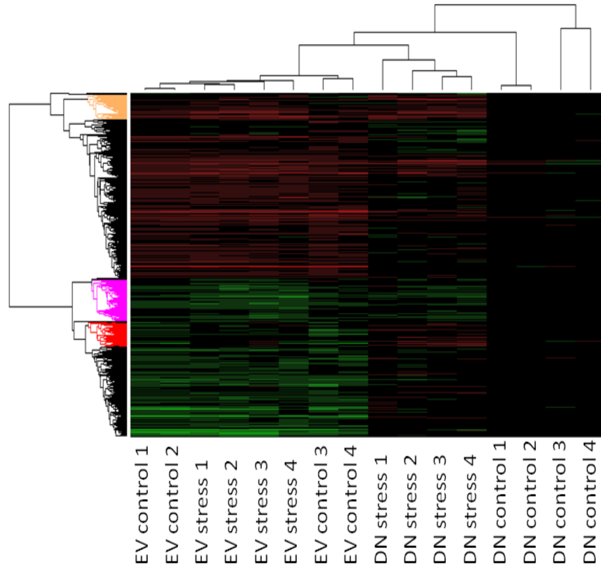

Fig. 3. Two-way hierarchical clustering in the list of 4075 probesets yielded by ANOVA analysis. Linkage method: Average, distance: Pearson correlation.

III. Results and Discussion

The integrative analysis firstly examined the predictive consistency between the integrated dataset and the results of the old dataset, in the comparison of the EV versus DN cell lines. This would set a checkpoint prior to the extensive utilization of the integrated dataset for translational purposes. After normalizing the data as described, statistical analysis, using t-test was employed (threshold p-value 0.05, FDR 0.1) in both datasets (integrated bearing 4 replicates for each condition and the initial with 3 replicates at each condition). The comparison between EV and DN_IRE1α classes was very high, above 90 %, supporting the legitimacy of the pooling. Then for the comparison between the 4 conditions, a similarly derived DE gene list (ANOVA FDR<0.1, p-value<0.05), yielded 4075 members) to steer further analysis.

The next step was the hierarchical clustering analysis (Fig. 3), performed as described above, which provides insight regarding the regulatory impact of the crosstalk between stress induction and the UPR in both (EV, DN_IRE1α) lines. The clustering analysis confirms the normalization approach we have adopted as it groups together as a solid cluster the DN_IRE1α stress category. This supports the notion of the pivotal role of IRE1α as a mater regulator, evoking a broad repertoire of molecular responses which fine-tunes UPR, something that can be clearly apprehended

TABLE I

Binding Sites Recognized by ATF6 and XBP1 Transcription Factors in the Promoters of the Three Tested Clusters

	Cluster 1	Cluster 2	Cluster 3	Vertebrate promoters
ATF6	56	54	54	16.8
XBP1	33	37	30	17.9

Values indicate the percentage of promoters in each cluster found to contain at list one of the indicated TF binding sites. The values in the last column indicate the percentage of vertebrate promoters found to contain the same binding site, under the same search criteria.

at the absence of this mechanism (DN_IRE1α) where the profile of the homeostatic response clusters all these stressors together.

From the heatmap illustration of the complete tree, three interesting sub-clusters are derived concerning IRE1α independent actions (Cluster 1 overexpression, Cluster2 underexpression, Cluster 3 substitution). Clusters 1 and 2 comprise genes regulated by stress in an IRE1α independent suggesting, putative targets of the PERK and ATF6 UPR regulators. Classical ER stress response genes like are members of these lists whereas the prioritized pathway analysis by StRAnGER highlights as important biological processes, functions congruent with the established pro-apoptotic, anti-proliferative, inflammatory character of the pathways related with these genes. Cluster 3 encompasses genes that could be associated with the hypersensitivity of the DN to the stresses or constitutively cleaved by IRE1. In accordance with this idea, there is 10% of the genes associated with the cell death pathways in this cluster. Then, in the DN, this control is disrupted and results in gene overexpression upon stress.

Having derived the gene sets of each of the aforementioned clusters, we searched the Cold Spring Harbor promoter database in order to extract their promoter sequences. Sequences from -700 up to +300, relative to the transcription start site, were analyzed for common Transcription Factor (TF) binding motifs, exploiting the MatInspector tool of Genomatix, which enables massive, prioritized screening of the promoter areas at search, combining both similarity and extent of membership criteria. The thresholds adopted targeted the identification of transcription factor binding areas with very high affinity (p-value<10^{-20}), to ensure specificity of interaction and at the same time the identification of modules with pronounced regulatory role for the clusters under test (>70% of the promoters). This initial round of search for TF binding sites revealed several elements for each cluster and we focused on those related to XBP1 and ATF6 dependent transcription. The results of this analysis are shown in Table I. Given that ATF6 and XBP1 are key regulators of the ER stress response at the level of transcription, it is interesting that *cis*-elements recognized by these two factors were found to a proportion of genes at each analyzed cluster. This finding supports the validity of our analysis and suggests that the groups of genes raised from statistical and cluster analyses represent true targets of the ER stress pathway.

IV. CONCLUSION

The present study represents an integrative computational analysis of transcriptomic data aiming to elucidate the molecular circuitry involved in ER stress response in glioblastoma cells. To this end, GO-based analysis on selected clusters as well as promoter analysis, gave solid evidence of molecular mechanisms possibly responding to XBP1 and/or ATF6 transcriptional signaling. Further analyses exploiting semantic similarity criteria are under way, in order accomplish the active molecular network of ER Stress Signaling in Glioblastoma.

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