

## CHAPTER 4

### CONCLUSIONS AND FUTURE PERSPECTIVES

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Protein-protein interactions play a key role in the normal homeostasis as well as in diseases. Thus, utilization of techniques that helps in understanding these interactions are extremely useful in the identification of novel interactions that has potential in understanding the pathogenesis as well as deliver important biomarkers of diagnostic/prognostic/therapeutic significance. The technique exploiting this interaction developed by us is called far-western approach [217]. Using the platform of far-western technology, we analysed CSF from leukemia samples towards identifying novel biomarkers for CNS leukemia for better treatment modalities for patients suffering from CNS leukemia. As an initial step, to identify the biomarker of importance, qualitative and quantitative representation of proteins from the lymphoblasts on the gel is essential. The high resolution two dimensional gel electrophoresis is one such powerful technique to profile the proteins towards good representation on the gel and is an indispensable tool in most of the cancer biomarker discoveries [306]. In any proteome based analysis including 2D gel electrophoresis, the crucial step is the sample preparation. We thus standardised the sample preparation for 2D-PAGE to maximally represent the proteins on the gel and for the subsequent far-western analysis. The differential precipitation of proteins by difference in the physico-chemical interaction of proteins to the chemicals makes the precipitation strategies a tool with limitations for better representation. In order to evade this limitation and to find out a better strategy for maximum protein representation on the gel, we compared the non-precipitation strategy of centricon method of concentration of proteins to different protein precipitation strategies [280]. The centricon concentration of lymphoblastic cell lysates gave maximum representation of proteins on the gel (with 1116 spots vs. 1036 in ethanol, 750 in Ac, 720 in MAc and 385 in PCI) compared to other conventional precipitation strategies and hence the same strategy was used for further far-western studies [280].

In the next stage of study, the reactivity of sequential CSF samples collected from the same ALL patients at different stages of the disease was tested towards the 2D profiled lymphoblastic proteins on the blot by the far-western approach as described by Menon et al., 2011 [217]. Comparison of CSF reactivity profiles of sequential samples from the same and

different patients showed consistent pattern of differential reactivity to CSF during presentation, remission and relapse towards few select lymphoblastic proteins. The differentially reactive lymphoblastic proteins were identified by mass spectrometry and the proteins were PFDN5 $\alpha$ , CIP29, ECH1 and PRDX6. These molecules have been shown to have a role in cancer development and the proteins PFDN5 $\alpha$ , CIP29 and ECH1 were shown to play a role in leukemia genesis (Table 3.8). The protein PFDN5 $\alpha$  is more reactive to CSF samples at remission or the patient is recovered from the disease. On the contrary, the reactivity to PFDN5 $\alpha$  was found to be low towards the CNS leukemia samples either at presentation or at CNS relapses or with high risk for developing CNS leukemia. Proteins such as CIP29, ECH1 and PRDX6 showed a pattern of more reactivity to presentation or CNS relapse samples and less reactivity to remission or following treatment CSF samples on 2D western blots.

Based on the differential reactivity profile towards CSF samples, from the ALL patients at different time points and the role in different cancers including leukemia, the identified proteins PFDN5 $\alpha$ , CIP29, ECH1 and PRDX6 were recombinantly expressed in bacterial system and purified in bulk quantity. The CSF reactivity profile to these purified proteins on the 1D blot were then compared to that on the 2D blot. Though these proteins showed differential reactivity to CSF on the 2D blots (wherein the proteins are represented from mammalian lymphoblasts), the bacterially expressed protein behaves differently except for PFDN5 $\alpha$ . The proteins CIP29, ECH1 and PRDX6 didn't show a pattern which is similar to that on the 2D blot. The difference in the reactivity profile towards the purified proteins except PFDN5 $\alpha$  may be due to the improper post-translational modification in the bacterial system as the bacterial systems do not have the same modifications on recombinantly expressed proteins. The CSF reactivity towards bacterially expressed PFDN5 $\alpha$  may not be directed against post-translational modifications. Thus further studies using mammalian expression systems have to be used for the analysis of CIP29, ECH1 and PRDX6.

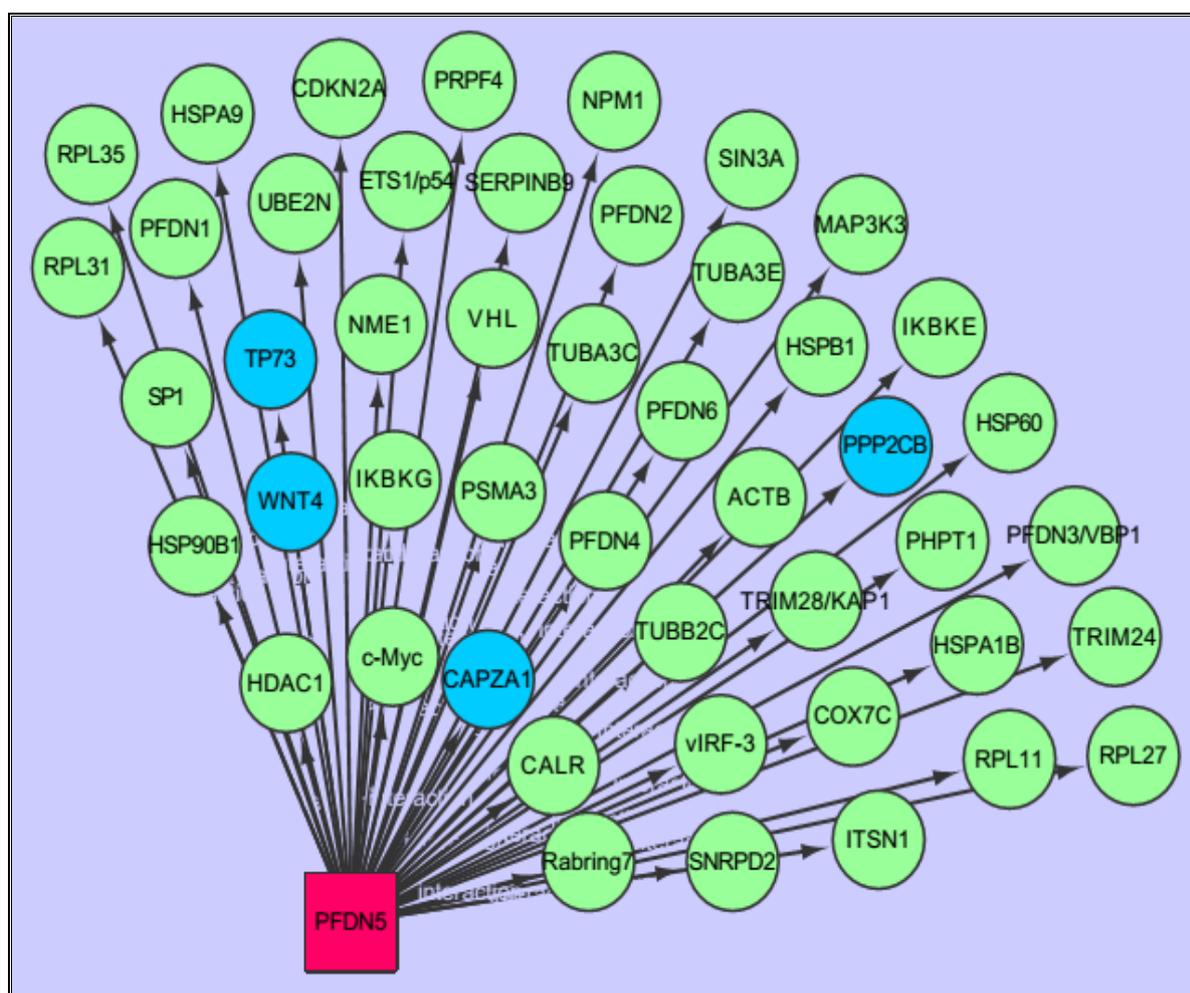
Among the recombinantly expressed purified proteins, only the purified PFDN5 $\alpha$  protein showed a pattern of differential reactivity to CSF samples which is similar to that observed on 2D far-western blots. The validation results thus indicate that the molecule identified on the basis of differential CSF reactivity observed on 2D-far-western blots is indeed PFDN5 $\alpha$ . This shows the importance of validation of candidate biomarkers to confirm its role in the concerned disease. From our results on PFDN5 $\alpha$  reactivity towards CSF samples from

presentation, remission and relapse, it is clear that PFDN5 $\alpha$  may serve as a potential prognostic marker for the underlying biochemical changes even when the patient is clinically asymptomatic for the CNS disease but later on diagnosed as CNS leukemia as judged by CNS cytology. Thus, even when the patient is clinically asymptomatic for the CNS disease, CSF reactivity analysis towards PFDN5 $\alpha$  could be used as a good tool to predict the underlying disease leading to clinical manifestations later. Therefore, development of a quantitative clinical evaluation strategy towards development of a prognostic marker has immense value in clinic.

With the intention of developing a quantification platform for prognosis and to further evaluate the CSF reactivity towards PFDN5 $\alpha$  in another system, we used ELISA method. Quantification of PFDN5 $\alpha$  reactivity using ELISA methodology followed the same reactivity pattern observed on far-western 2D blots and 1D blot using purified protein suggesting the ELISA method could be used for prognostic evaluations of patient's clinical status. Thus, with the use of techniques such as far-western proteomics, western blotting and ELISA, we have validated the potential of CSF reactivity to PFDN5 $\alpha$  in the prognosis of CNS disease in ALL. In other words, when the patient is fighting fit and responds well to the therapy and the clinical status is normal, the CSF reactivity towards PFDN5 $\alpha$  protein is high ( $>0.05$ ). On the same token, when the patient develops the CNS disease or relapse later during therapy, the reactivity to PFDN5 $\alpha$  is significantly low ( $<0.05$ ). However, during the development of CNS leukemia, where the CNS cytology shows no blast cells in the CSF and the clinical diagnosis of CNS leukemia is not possible, based on the low reactivity closer to 0.05 or less observed herein indicates a high risk of developing the CNS disease (Figure 3.15 and 3.17). Thus, PFDN5 $\alpha$ 's differential reactivity profile during remission and relapse could be used as a prognostic indicator for the underlying biochemical pathogenesis that happens well before the appearance of clinical manifestation of CNS disease in ALL patients. Further analysis using more patient samples with CNS involvement are required to further validate and to refine the cut off value of  $<0.05$  to be more precise. Thus monitoring this reactivity could help in starting the therapy well before the clinical manifestation of the disease. Therefore, prophylactic administration of CNS chemotherapy can be made more judicious that patient burden may be reduced further as a result of chemotherapy.

Based on the far-western and validation studies, it is clear that the low CSF reactivity to PFDN5 $\alpha$  below certain level ( $<0.05$ ) is an indication for CNS involvement in ALL patients or

its early prediction in patients. But the importance of low CSF reactivity to PFDN5 $\alpha$  in CNS leukemia pathogenesis can be revealed only after the identification of the interacting partner of PFDN5 $\alpha$  in the CSF. The interacting molecule can be another protein or metabolite or immunoglobulins or their fragments or cytokines or chemokines etc. But the interesting fact is that the low interaction or in other words the low level/down regulation of the interactor seems to be important for the pathogenesis of CNS leukemia. For the identification of this unknown interacting partner, a proper experimental strategy should be developed. Meanwhile bioinformatics analysis was attempted to identify the interacting molecules of PFDN5 $\alpha$ . The major limitation of bioinformatics analysis is that only reported molecules of protein nature can be assumed as the interacting partner of PFDN5 $\alpha$ . Some of the reported high scored interacting partners of PFDN5 $\alpha$  from different data bases like STRING, BioGrid, IntAct, I2D, PPI Finder and APID are represented together in the figure 4.1.



**Figure 4.1** Protein interaction network of PFDN5 $\alpha$ . The proteins represented in blue have role in metastasis when down regulated.

Majority of the interacting proteins are other prefoldin subunits, heat shock proteins, actin/tubulin proteins, ribosomal proteins etc. Though the over expression of most of the interacting proteins was found to be responsible for cancer progression, invasion and metastasis; proteins like CAPZA1, TP73, WNT4 and PPP2CB were metastatic and invasive under down regulated mode (represented in blue in Figure 4.1). So these proteins in CSF may correlate with the CNS relapse with low reactivity to PFDN5 $\alpha$  but their presence in CSF has not yet proved. The high levels of these proteins during treated or clinically better condition of the patient may be due to treatment induced up regulation or due to immune response signal towards these foreign cells and resultant over expression. In addition to these protein interactors, there are chances that the interacting molecule may be of non-protein in nature or any secretory molecule which is not reported yet or metabolites. Currently there are no data bases available to find out the non- protein or metabolite partners of proteins and also for the CSF proteins under leukemia situation and these limitations makes the prediction about the interacting molecule using bioinformatics tools is a difficult task. Therefore, it is extremely important to find out the interacting molecule having differential reactivity towards PFDN5 $\alpha$  under different clinical conditions of CNS leukemia patient like presentation, relapse and remission using experimental systems for better understanding of disease pathology, correct prognosis and appropriate treatment option selection.

The major outcomes of the study are follows:

- ❖ Developed a platform for the qualitative maximum representation of lymphoblastic proteins on the 2D gel
- ❖ Among the different sample preparation strategies experimented, maximum protein representation was detected in centricon concentration strategy in which no protein precipitation is involved.
- ❖ The representation of proteins in the higher molecular weight region (50-250kDa) was also found to be high in centricon concentration.
- ❖ Based on the results of the comparative analysis on different sample processing strategies, centricon concentration of cell lysate was chosen as a method of sample preparation for 2D gel electrophoresis and further studies.

- ❖ Lymphoblastic proteins showed differential reactivity to CSF samples collected from a heterogeneous population of ALL patients at different age, sex, blast levels and time points such as CNS involvement, remission and relapse, the pattern of CSF reactivity with respect to selected molecules are consistent between patients despite the heterogeneity.
- ❖ The proteins which showed a consistent pattern of differential CSF reactivity were identified by mass spectrometry
- ❖ Protein which showed high reactivity under remission is PFDN5 alpha.
- ❖ CIP29, ECH1 and PRDX6 are more reactive under presentation/relapse condition compared to the remission as per 2D western blot analysis.
- ❖ There is an inverse correlation in the reactivity pattern between proteins reacting at remission vs. relapse/presentation that they may have a prognostic importance in CNS leukemia as per 2D western blot analysis.
- ❖ Proteins PFDN5 $\alpha$ , CIP29, ECH1 and PRDX6 were selected for validation and recombinantly expressed and purified from bacterial system using Ni-Agarose beads
- ❖ CSF reactivity towards the purified proteins were analysed and compared to that on the 2D blots. PFDN5 $\alpha$  was the only protein showed a differential reactivity to CSF samples from different clinical statuses of the patients as that on the 2D far western blot. The reactivity profile to all other proteins was found to be not consistent to that seen on 2D far western blots.
- ❖ Based on the validation results, PFDN5 $\alpha$  was selected for ELISA quantification and to further validate the reactivity pattern seen on western blots such that a strategy for quantitative evaluation of CSF reactivity in clinic could be developed.
- ❖ ELISA quantification also showed a pattern of CSF reactivity to PFDN5 $\alpha$  which is similar to that on the 2D blots and towards the purified protein. With the limited number of five CNS leukemia samples analysed, the average CSF reactivity to PFDN5 $\alpha$  when CNS leukemia is seen is found to be  $0.026 \pm 0.007$ . Most importantly, all the CNS leukemia samples used in the analysis showed low reactivity i.e.,  $\leq 0.50$  OD to PFDN5 $\alpha$  (Fig: 3.16).

- ❖ These results point to the fact that low reactivity to PFDN5 $\alpha$  gives an indication for relapse in the patient and a judicious treatment could be planned to reduce the side effects of currently used prophylactic therapy. However, this requires further validation with large number of patient samples is essential.

### Future perspective

Differential reactivity of PFDN5 $\alpha$  to CSF from different clinical stages of presentation, remission and relapse indicates a significant role of PFDN5 $\alpha$  in the prognosis of leukemia. Moreover, identification of PFDN5 $\alpha$  interacting partner in the CSF of leukemic patients is important for the further understanding of the leukemia pathogenesis. Reportedly, PFDN5 $\alpha$  protein has a role in regulating other molecules which are involved in leukemogenesis like c-Myc, Wnt, Proteasome subunits etc. and these functions makes it as a good therapeutic target also. Notably, mutation of Alanine to Arginine at the 157<sup>th</sup> position abrogates the function of PFDN5 $\alpha$  and is common in 50-60% of leukaemia and lymphoma cases making it difficult to control the oncogenes c-Myc and Wnt. Hence, the existence of a fully functional PFDN5 $\alpha$  protein in the cell is important for its proper functioning. Therefore, expression of functional PFDN5 $\alpha$  in the cell could be crucial in the therapy against leukemia. Additionally reactivity to PFDN5 $\alpha$  in the blood also is important towards development of a minimally invasive diagnostic perspective.