The First Reported Mutations in the Pituitary Tumor-Transforming gene (PTTG) Binding Factor (PBF)

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Introduction

PBF is an ubiquitous glycoprotein which is over-expressed particularly in endocrine and endocrine -related cancers. Previously classified as a proto-oncogene, ten substitution-missense and one nonsense mutations of PBF have now been reported in tumours from patients with ovarian, prostate and colorectal cancers via the COSMIC database, suggesting PBF may in fact be an oncogene.

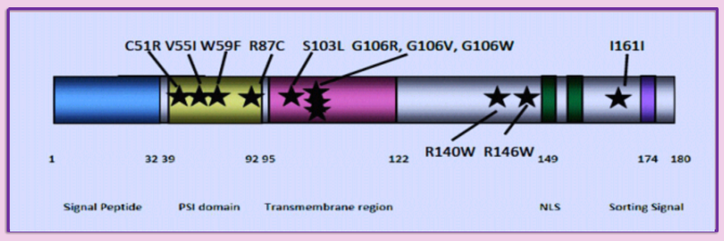

Figure 1. Schematic diagram of the PBF structure, with the 11 amino acid residue changes denoted by asterisks. Data taken from COSMIC database.

Eight substitution residues are located in the PSI domain and transmembrane region of PBF, while two remaining mutants are close to the nuclear localisation signal (NLS).

Hypotheses

- Amino acid substitutions may affect the stability and proto-oncogenic actions of PBF, leading to a re-classification of PBF as an oncogene.
- The PSI domain and NLS may be critical functional domains of PBF function.

Prediction of protein function

Following COSMIC database searches, nucleotide sequences of wild-type and mutated PBF were assessed via the I-TASSER server for reconstruction of 3-dimensional protein structures. (Figure 2A and Figure 2B)

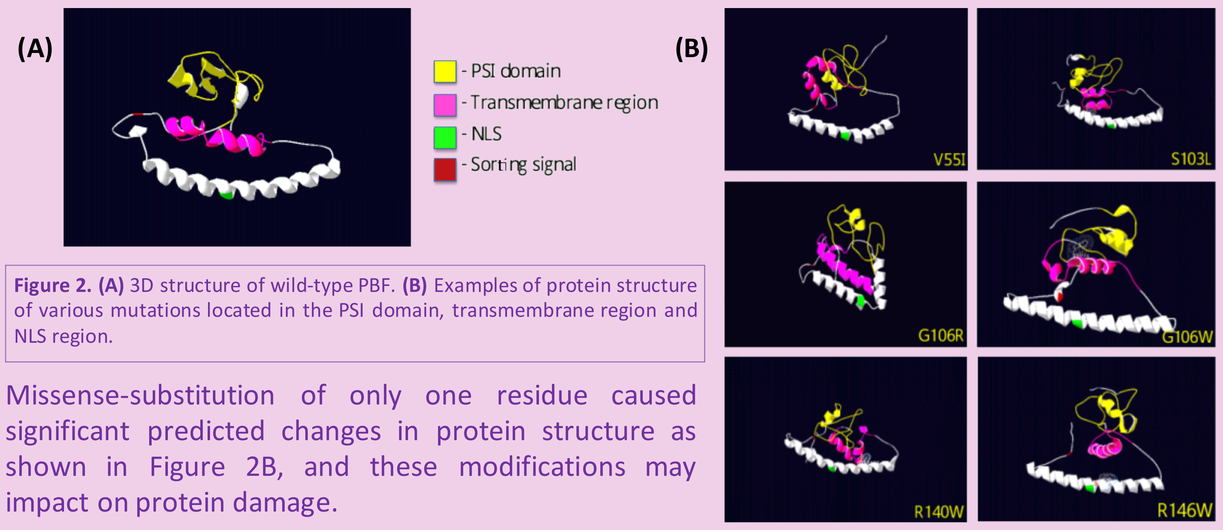

Figure 2. (A) 3D structure of wild-type PBF. **(B)** Examples of protein structure of various mutations located in the PSI domain, transmembrane region and NLS region.

Missense-substitution of only one residue caused significant predicted changes in protein structure as shown in Figure 2B, and these modifications may impact on protein damage.

User Input	ENSP	Pos	Ref	Subst	Prediction	SIFT Score	Median Information Content	# Seqs
ENSP00000328325,G106R	ENSP00000328325	106	G	R	DAMAGING	0.01	2.8	49
ENSP00000328325,G106V	ENSP00000328325	106	G	V	TOLERATED	0.12	2.8	49
ENSP00000328325,G106W	ENSP00000328325	106	G	W	DAMAGING	0	2.8	49
ENSP00000328325,C51R	ENSP00000328325	51	C	R	DAMAGING	0	2.8	49
ENSP00000328325,V55I	ENSP00000328325	55	V	I	DAMAGING	0.05	2.8	48
ENSP00000328325,W59F	ENSP00000328325	59	W	F	DAMAGING	0.02	2.8	49
ENSP00000328325,R87C	ENSP00000328325	87	R	C	DAMAGING	0	2.8	49
ENSP00000328325,S103L	ENSP00000328325	103	S	L	DAMAGING	0.01	2.8	49

Figure 3. Table illustrated the SIFT score taken from SIFT website, which predicted protein damage in all substitutions except G106V. Scores less than 0.05 are expected to affect protein function.

Sorting Intolerant From Tolerant (SIFT) assessment, based on sequence homology and physical properties of amino acids, was applied for all mutations to predict whether specific amino acid substitutions might alter protein function (Figure 3). All mutations except G106V were predicted to elicit protein damage, so all ten 'damaging' substitutions were investigated in vitro.

PBF glycosylation

To determine the effect of all mutations, COSMIC database amino acid substitutions were recapitulated in pcDNA3-PBF, and tagged with HA at the C-terminus. The R140W mutant was FLAG-tagged at the N-terminus as it could not be detected in Western blotting when HA tagged at the C-terminus. (Figure 4A and Figure 4B)

Figure 4. (A) Schematic of the full PBF protein highlighting various protein domains. **(B)** Full length PBF cDNA was tagged at the N-terminus with a FLAG tag and at the C-terminus with a HA moiety.

Following transfection of COS-7 cells, principal PBF bands at ~25, 30 and 37 kDa were detected via Western blotting analysis, which represent differentially glycosylated isoforms. In addition, potential oligomers at ~50 and 75 kDa were apparent. Mutations of C51R and G106R reduced oligomerisation and glycosylation. In addition, there was no PBF band detection for R140W, so a FLAG tagged R140W plasmid was created.

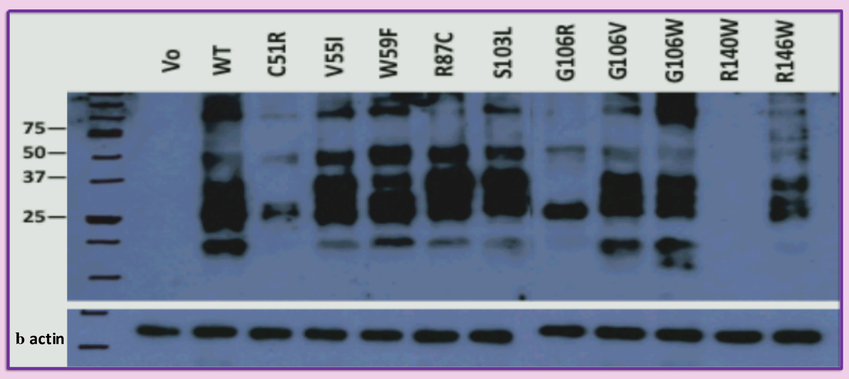

Figure 5. COS7 cells were transiently transfected with vector only (VO), wild-type (WT) PBF-HA and all HA tagged PBF mutants. Following protein extraction, PBF bands were detected by Western blotting with a HA antibody. The figure illustrates Western analysis of VO, WT PBF alongside all mutations.

PBF stability

Following 24 hours of transfection, Anisomycin, which inhibited protein synthesis, was added to MCF7 cells prior to Western blotting. Protein was harvested at 0, 12 and 24 hours, respectively. After Western analysis, densitometry was performed to evaluate protein resilience, enabling the half life of each mutant to be calculated. (Figure 6A and 6B)

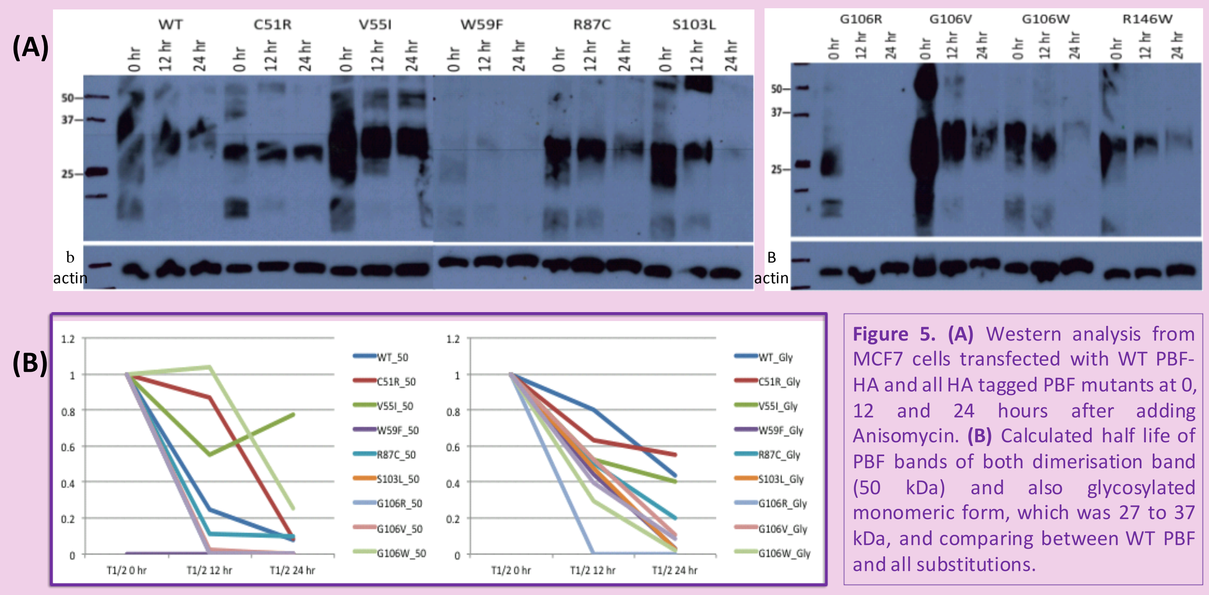

Figure 5. (A) Western analysis from MCF7 cells transfected with WT PBF-HA and all HA tagged PBF mutants at 0, 12 and 24 hours after adding Anisomycin. **(B)** Calculated half life of PBF bands of both dimerisation band (50 kDa) and also glycosylated monomeric form, which was 27 to 37 kDa, and comparing between WT PBF and all substitutions.

The half life study in MCF7 breast cancer cells revealed that C51R mutation had a considerable increase in protein stability, with a half life of more than 24 hours, compared to wild type (half life approximately 22 hours), whereas V55I, W59F, G106R, G106V, G106W and R146W mutants were less stable, ranging from approximately 6 hours to 12 hours. Interestingly, different tumour mutations at residue 106 revealed very different biochemical properties of the protein, with G106R showing significantly reduced glycosylation, dimerisation and half life, whereas G106V and G106W demonstrated wild type levels of dimerization, and with G106V having 50% greater stability than G106W.

Proliferation

To identify the effect of amino acid residue changes on cell proliferation, TPC cells were transfected for 24 hours, reseeded into 96-well plates and then incubated for 24 hours before performing proliferation assays.

Figure 6. TPC cells transfected with WT PBF and all untagged PBF mutants were analysed by proliferation assays using a reagent containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt (MTS) and an electron coupling reagent, which was phenazine ethosulfate (PES). Following incubation, the absorbance of the cells was measured at 490 nm to determine the number of viable cells comparing WT and in vivo mutants of PBF. Statistical analysis was performed with one way ANOVA test. N=3.

The assay revealed that most mutations have pro-proliferative effect on the cells. However, only substitution of V55I in the PSI domain, and R140W, close to the NLS, harboured statistically significant increases in cell proliferation when compared with wild-type PBF. Mutants C51R and G106V (circled in red) were borderline significant.

Conclusions

- The first reported mutations of PBF are potentially oncogenic in vivo based on our initial biochemical characterisation of three-dimensional protein structure, glycosylation, dimerisation, stability and proliferation assays.
- C51R and G106R reduced oligomerisation and glycosylation in vitro.
- Mutation of the cysteine at residue 51 to an arginine provides increased protein stability of PBF, while substitution of arginine at residue 106 made the PBF protein less stable.
- De novo mutations within the PSI domain and proximal to the NLS induce significant cell proliferation.
- Overall, the first described mutations in the proto-oncogene PBF suggest that whilst rare, mutations in PBF may drive proliferation, uncovering a potentially new mechanism in human cancer.

References

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