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Hotspot activating *PRKD1* somatic mutations in polymorphous low-grade adenocarcinomas of the salivary glands

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AUTHOR CONTRIBUTION

IW, LN, JDM, BAC, BW, PCB and JSR-F conceived the study and supervised the work. IW, LDRT, SIC, RRS, AS, EBS, IF, AA, CH, JW, RB, MC-S-Y, CJH, CAN, YHW, NK and BPR provided samples. IW, BP-O, YHW and NK reviewed the cases. Sample processing, Sanger sequencing and amplicon sequencing were performed by SP, NF, ACP, CH and F-FL. Massively parallel RNA sequencing and whole exome sequencing were carried out by AV, JDM and PCB. RNA sequencing analysis was performed by CKYN and RL. Whole exome sequencing analysis was performed by DW, CKYN, NJH, RL, NB and LM. JDM, PCB and JSR-F coordinated the bioinformatic analyses. *In silico* protein structure analysis was performed by SP, LGM, JA, TK, BGW and PCB. Functional *in vitro* experiments were carried out by LGM and BW. RAS carried out immunohistochemical analysis. JSR-F and CM interpreted the immunohistochemical results. CKYN, KCC and PCB performed statistical analyses. BW and JSR-F wrote the first draft of the manuscript, which was initially reviewed by IW, SP, LGM, DW, LN, BPR and PCB. All authors edited and approved the final draft.

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Abstract

Polymorphous low-grade adenocarcinoma (PLGA) is the second most frequent type of malignant tumor of the minor salivary glands. We identified a *PRKD1* p.Glu710Asp hotspot mutation in 72.9% of PLGAs but not in other salivary gland tumors. Functional studies demonstrated that this kinase-activating mutation likely constitutes a driver of PLGAs.

Polymorphous low-grade adenocarcinoma (PLGA) is the second most common intra-oral malignant salivary gland tumor, preferentially affecting the minor salivary glands[1]. This tumor occasionally originates in other anatomical sites, including the lungs and breast[2]. PLGAs are characterized by cytologic uniformity and histologic diversity[1]. Despite their infiltrative growth pattern and tendency for perineural invasion, PLGAs have a relatively indolent clinical course, with lymph node metastasis in up to 29% of cases and rare distant metastasis[3,4]. Owing to the large spectrum of histologic appearances, the differentiation of these tumors from more aggressive forms of salivary gland cancers is challenging, especially in small diagnostic specimens.

Recent studies have demonstrated that distinct types of malignant salivary gland tumors, including adenoid cystic carcinoma (AdCC), mucoepidermoid carcinoma and secretory carcinoma, are driven by highly-recurrent specific somatic genetic alterations (*e.g.* fusion genes)[5]. Like PLGAs, these tumor types can also originate in other anatomical sites,

including the lungs and breast[2]. We therefore reasoned that PLGAs might be driven by a highly-recurrent somatic genetic alteration.

To test this hypothesis we subjected three consecutive PLGAs to massively parallel RNA-sequencing and whole exome sequencing, and a separate set of three PLGAs to whole exome sequencing (Supplementary Table 1). RNA-sequencing did not reveal any recurrent in-frame fusion genes (Supplementary Table 2). By contrast, whole exome sequencing identified two somatic heterozygous single nucleotide variants (SNVs) in the *PRKD1* gene, c.2130A>T and c.2130A>C, affecting a highly conserved amino acid residue in the catalytic loop (Fig. 1a) and resulting in the same amino acid substitution p.Glu710Asp (E710D) in five of six cases (Fig. 1b, Supplementary Table 1, Supplementary Fig. 1). RNA-sequencing demonstrated that the mutant alleles were expressed (Supplementary Table 1, Supplementary Fig. 2). No additional recurrent somatic SNVs or insertions/deletions were detected (Supplementary Table 3). We therefore subjected a validation cohort of 53 PLGAs to Sanger sequencing and targeted amplicon sequencing of *PRKD1* exon 15, and confirmed the presence of somatic c.2130A>T and c.2130A>C mutations in 41.5% and 30.2% of PLGAs, respectively (Fig. 1b, Supplementary Fig. 3, Supplementary Table 4). Taken together, 43 of 59 (72.9%) PLGAs tested harbored the *PRKD1* p.Glu710Asp mutation. Detailed pathologic analysis of PLGAs with mutated and wild-type *PRKD1*, however, did not reveal distinct histologic features that would differentiate the two groups (data not shown). PLGAs with and without the *PRKD1* p.Glu710Asp mutation were found to express PRKD1 protein (Fig. 1c, Supplementary Figs. 2b and 2c), suggesting that in a way akin to other mutation-activated oncogenes[6], PRKD1 expression is more pervasive than the presence of a *PRKD1* hotspot mutation.

PRKD1 is a serine/threonine kinase that plays a role in cell adhesion, cell migration, vesicle transport and cell survival[7]. We found the *PRKD1* gene to be mutated in 2.2% of cancer samples included in The Cancer Genome Atlas (TCGA) and other published datasets available on cBioPortal (http://www.cbioportal.org)[8], but none of these cancers harbored the *PRKD1* c.2130A>T or c.2130A>C (p.Glu710Asp) mutations, suggesting that these SNVs may be unique to PLGAs (Figs. 1d and 1e). In addition, this novel *PRKD1* mutation has not been reported in the Catalog of Somatic Mutations in Cancer[9] (v68; data not shown). To determine whether the *PRKD1* p.Glu710Asp mutation would be pathognomonic of PLGAs, we investigated the presence of this mutation in a series of 311 tumors of the salivary glands (186 malignant and 125 benign lesions), of which 12 were PLGAs. The *PRKD1* p.Glu710Asp mutation was found in 8 of the PLGAs, but not in any of the other salivary gland tumors studied (Fig. 1f, Supplementary Methods, Supplementary Table 5). Therefore, our findings demonstrate that the *PRKD1* p.Glu710Asp hotspot mutation, akin to *CRTC1-MAML2* and *MYB-NFIB* fusion genes defining subsets of mucoepidermoid carcinomas and AdCCs respectively[5], defines a subset of PLGAs of the salivary glands, and may be useful as an ancillary diagnostic marker to differentiate PLGAs from other forms of salivary gland tumors. In addition, the presence of the *PRKD1* p.Glu710Asp mutation was significantly associated with metastasis-free survival of patients with malignant salivary gland tumors (Fig. 1g, Supplementary Methods, Supplementary Table 6), suggesting that this mutation may be useful in the molecular prognostic work-up of salivary gland cancers.

The p.Glu710Asp hotspot mutation affects the catalytic loop HCDLKPEN of the PRKD1 protein kinase domain (Fig. 1a), and was predicted to be pathogenic by independent mutation function assessment algorithms (Supplementary Table 3). Although the crystal structure of PRKD1 is not yet solved, homology modeling suggested that the p.Glu710Asp mutation can either interfere with ADP binding by altering coordination with Mg^{2+} or impact enzyme kinetics, as it is positioned in between the ATP donor binding site and the putative proton acceptor (Fig. 2a; Supplementary Fig. 4a). The Glu (E) residue is highly conserved among the CAMK kinases, however an Asp (D) residue is frequently observed within the native structures of AGC kinases, suggesting that this mutation is likely active but may induce a functional shift (Supplementary Fig. 4b). Consistent with these protein structure predictions, in a cell-free *in vitro* kinase assay p.Glu710Asp-mutant PRKD1 displayed significantly increased transphosphorylation of the serine-threonine substrate CREBtide and elevated autocatalytic activity than wild-type PRKD1 (Fig. 2b; Supplementary Fig. 5a). Forced expression of wild-type and p.Glu710Asp-mutant PRKD1 in embryonic kidney epithelial and non-malignant breast epithelial cell lines (Supplementary Fig. 5b) revealed increased phosphorylation of Ser738/742 within the activation loop and Ser910 in the autocatalytic site of the mutant PRKD1 relative to wild-type in all subcellular compartments (Fig. 2c; Supplementary Fig. 5c), in agreement with the higher trans- and autocatalytic kinase activity detected in the cell-free kinase assay.

Previous studies have suggested that PRKD1 activation reduces cell migration and invasion, but promotes the growth of epithelial cells[7]. Consistent with this notion, forced expression of p.Glu710Asp-mutant PRKD1 in conventional *in vitro* models resulted in reduced cell migration, despite an increase in cell viability (Supplementary Fig. 6). To ascertain whether the *PRKD1* p.Glu710Asp hotspot mutation would affect the growth and glandular architecture of epithelial cells, we forced the expression of wild-type and p.Glu710Asp-mutant PRKD1 in two non-malignant breast epithelial cell lines, MCF10A and MCF12A, grown in three-dimensional cultures[10]. These cells were chosen given that PLGA cell lines and non-malignant models of minor salivary gland cells are not available and that, albeit rarely, primary PLGAs have been reported in the breast[2]. Forced expression of the p.Glu710Asp-mutant PRKD1 changed the hollow spheroid acinar-like structures formed by parental or empty vector-transfected MCF10A and MCF12A cells into larger, coalescent structures, with filled lumens and irregular contours not uncommonly displaying infiltrating edges, a phenotype consistent with that induced by the forced expression of other oncogenes in this model system[10,11](Fig. 2d). Taken together, our findings demonstrate that the somatic *PRKD1* p.Glu710Asp kinase domain hotspot mutation is likely activating and may confer a neoplastic advantage to epithelial cells.

We have identified somatic *PRKD1* p.Glu710Asp activating hotspot mutations in the majority of PLGAs, which may be employed as an ancillary molecular marker to differentiate PLGAs from their more aggressive mimics, including AdCCs. *PRKD1* emerges as a new cancer gene, which likely constitutes a driver of PLGAs and may potentially be exploited as a diagnostic aid and therapeutic target. Our study emphasizes that investigating the genetic underpinnings of rare cancer types can lead to the identification of novel oncogenic drivers and help the development of a molecular taxonomy of cancers[12].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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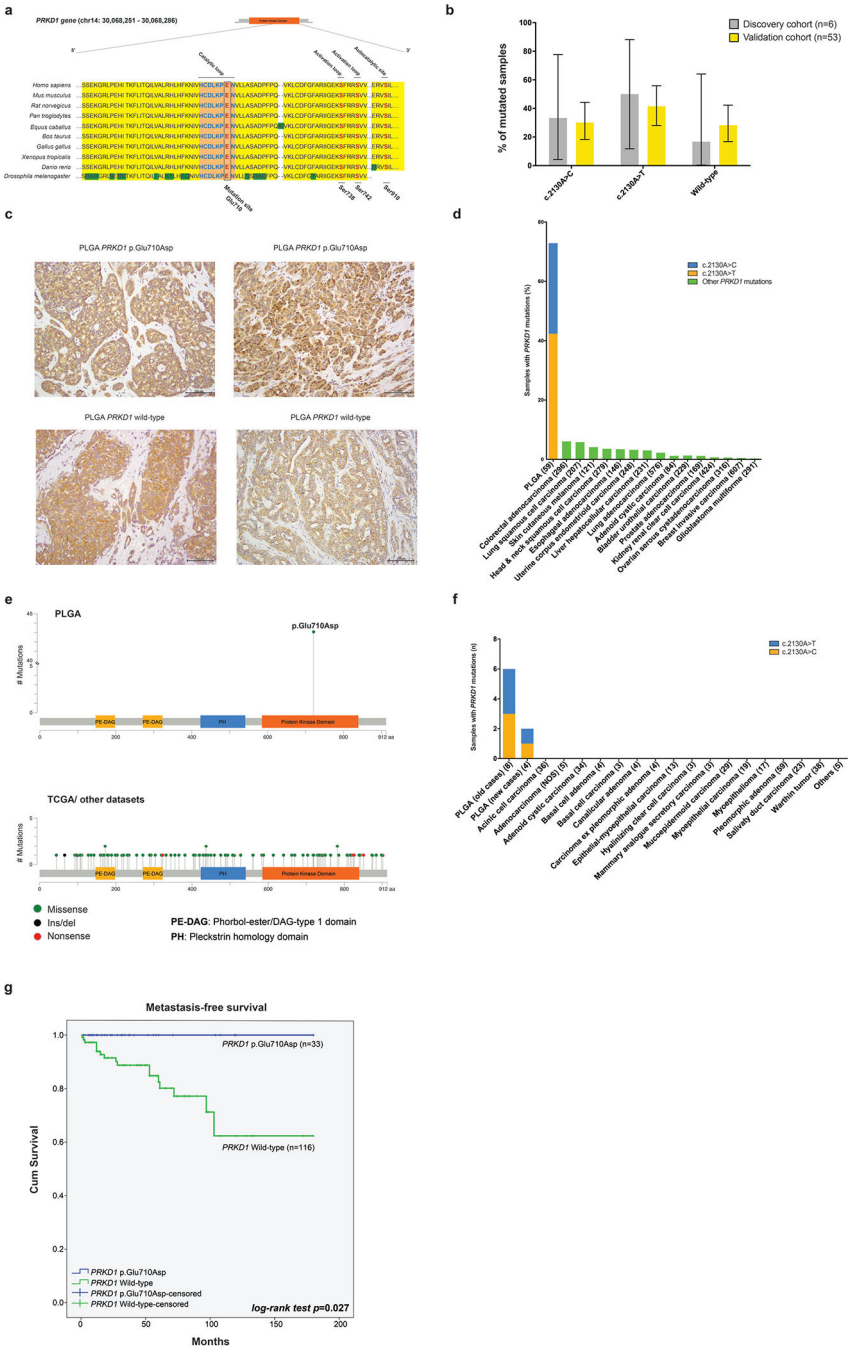

Figure 1.

PRKD1 mutations in PLGAs and other cancer types. (**a**) Multiple sequence alignment of the catalytic and activation loops within the PRKD1 kinase domain. Conserved and non-conserved amino acids are highlighted in yellow and green, respectively. (**b**) Frequencies of the *PRKD1* c.2130A>T and c.2130A>C (p.Glu710Asp) mutations in the discovery and validation PLGA cohorts. Error bars show 95% confidence intervals. (**c**) PRKD1 protein expression assessed by immunohistochemistry in *PRKD1* mutant and wild-type PLGAs. (**d**) *PRKD1* mutational frequencies in PLGAs and in other cancer types from published datasets

available from cBioPortal[8]. (**e**) Domain structure of the *PRKD1* gene and the mutations identified in PLGAs (top) and in other cancer types from published datasets (bottom) available from cBioPortal[8]. (**f**) *PRKD1* mutational frequencies in 12 PLGAs and in 299 salivary gland tumors of other histologic types. (**g**) Metastasis-free survival of 149 patients with malignant salivary gland tumors according to *PRKD1* mutation status.

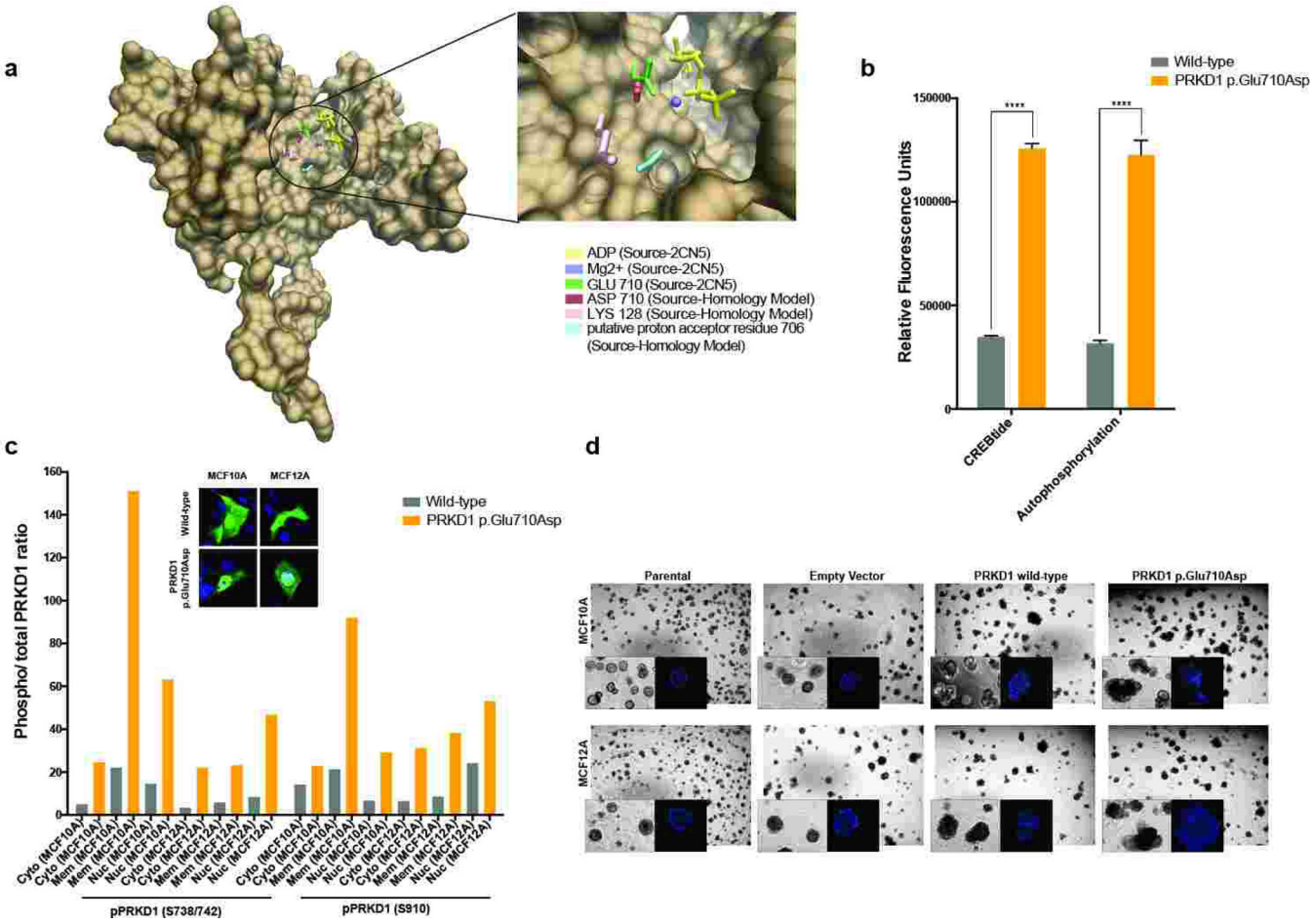

Figure 2.

Functional analysis of the *PRKD1* p.Glu710Asp mutation. (**a**) Surface render of a homology model for wild-type and p.Glu710Asp-mutant PRKD1 onto the structure of CHEK2 places the substitution in the midst of the active site between the ATP-binding pocket and the putative proton acceptor. (**b**) Cell-free *in vitro* kinase assay determining transphosphorylation of the serine-threonine substrate CREBtide and the autocatalytic activity of wild-type (grey) and p.Glu710Asp-mutant (orange) PRKD1. ****, Holm-Šídák-adjusted-P<0.0001, multiple t-test. (**c**) Subcellular distribution of wild-type (grey) and p.Glu710Asp-mutant PRKD1 (orange) in non-malignant breast epithelial MCF10A and MCF12A cells, and Ser738/S742 and Ser910 PRKD1 phosphorylation in distinct subcellular compartments. Cyto, cytoplasm; Mem, membrane; Nuc, nuclear; wt, wild-type. (**d**) Impact of wild-type and p.Glu710Asp-mutant PRKD1 expression on growth and glandular architecture of MCF10A and MCF12A cells grown in three-dimensional basement membrane cultures.