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Chromosome 9p copy number gains involving PD-L1 are associated with a specific proliferation and immune-modulating gene expression program active across major cancer types

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Abstract

Background: Inhibition of the PD-L1/PD-1 immune checkpoint axis represents one of the most promising approaches of immunotherapy for various cancer types. However, immune checkpoint inhibition is successful only in subpopulations of patients emphasizing the need for powerful biomarkers that adequately reflect the complex interaction between the tumor and the immune system. Recently, recurrent copy number gains (CNG) in chromosome 9p involving PD-L1 were detected in many cancer types including lung cancer, melanoma, bladder cancer, head and neck cancer, cervical cancer, soft tissue sarcoma, prostate cancer, gastric cancer, ovarian cancer, and triple-negative breast cancer.

Methods: Here, we applied functional genomics to analyze global mRNA expression changes associated with chromosome 9p gains. Using the TCGA data set, we identified a list of 75 genes that were strongly up-regulated in tumors with chromosome 9p gains across many cancer types.

Results: As expected, the gene set was enriched for chromosome 9p and in particular chromosome 9p24 (36 genes and 23 genes). Furthermore, we found enrichment of two expression programs derived from genes within and beyond 9p: one implicated in cell cycle regulation (22 genes) and the other implicated in modulation of the immune system (16 genes). Among these were specific cytokines and chemokines, e.g. CCL4, CCL8, CXCL10, CXCL11, other immunoregulatory genes such as IFN-G and IDO1 as well as highly expressed proliferation-related kinases and genes including PLK1, TTK, MELK and CDC20 that represent potential drug targets.

Conclusions: Collectively, these data shed light on mechanisms of immune escape and stimulation of proliferation in cancer with PD-L1 CNG and highlight additional vulnerabilities that may be therapeutically exploitable.

Keywords: Cancer, Immunotherapy, PD-L1, DNA copy number alterations, Chromosome 9p gain

Background

Programmed cell death 1 (*PD-1*)/PD-ligand1 (*PD-L1*) checkpoint inhibition has emerged as a promising treatment strategy in different advanced and often otherwise untreatable tumors in a growing number of cancer types. In this context immunohistochemical (IHC) evaluation of *PD-L1* protein expression has been proposed as a biomarker that predicts treatment efficacy. However, different IHC-antibody clones used for *PD-L1* detection and different evaluation methods including different cutoff values to distinguish 'positive' from 'negative' tumors introduce a substantial variance in the determination of *PD-L1* status. These ambiguities and the complex interplay of cancer-cell-inherent, microenvironment-associated and host-related factors [1] advocate further investigation of the

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Budczies *et al. BMC Medical Genomics* (2017) 10:74

Page 2 of 8

underlying biology to identify additional biomarkers that aid current stratification strategies.

PD-L1 copy number gains (CNG) are prevalent in significant subsets of different cancers including triple negative breast cancer [2], Hodgkin's lymphoma [3], cancer of unknown primary [4], NSCLC [5], SCLC [6], and gastric cancer [7]. In particular, Hodgkin's lymphomas harboring *PD-L1* CNG were reported to exhibit increased *PD-L1* expression and to respond well to immune checkpoint inhibition [3]. Adding to the growing body of evidence, enabled by The Cancer Genome Atlas (TCGA) a recent pan-cancer analysis of almost 10,000 tumors showed that *PD-L1* CNG were abundant in many cancer types with both focal and non-focal gains typically occurring at frequencies between 2% and 10% [8]. In this study, we identified a 7.8-Mbp region of 38 genes located in chromosome 9p24 (core amplified region) that is co-amplified with *PD-L1* in more than 80% of tumors with focal *PD-L1* gains across 22 cancer types. The core amplified region includes the genes *PD-L1*, PD-ligand 2 (*PD-L2*) and Janus kinase 2 (*JAK2*). Following up on these findings, we here show that chromosome 9p copy number gains are associated with specific mRNA expression changes enriched for genes implicated in cell cycle regulation and immune cell response.

Methods

PD-L1 copy number alteration (GISTIC) and genome-wide mRNA expression data (RNAseq v2) of the following 21 cancer types profiled in the TCGA project were obtained from the cBioPortal [9]: Bladder Urothelial Carcinoma (BLCA), Breast invasive carcinoma (BRCA), Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), Colorectal adenocarcinoma (COADREAD), Glioblastoma multiforme (GBM), Head and Neck squamous cell carcinoma (HNSC), Kidney renal clear cell carcinoma (KIRC), Kidney Renal Papillary Cell Carcinoma (KIRP), Brain Lower Grade Glioma (LGG), Liver hepatocellular carcinoma (LIHC), Lung adenocarcinoma (LUAD), Lung squamous cell carcinoma (LUSC), Ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), Pheochromocytoma and Paraganglioma (PCPG), Prostate Adenocarcinoma (PRAD), Sarcoma (SARC), Skin Cutaneous Melanoma (SKCM), Stomach adenocarcinoma (STAD), Papillary Thyroid Carcinoma (THCA), and Uterine Corpus Endometrial Carcinoma (UCEC). All TCGA data were freely available without restrictions on their use in publications (https://cancergenome.nih.gov/publications/publicationguidelines).

PD-L1 copy numbers alterations were evaluated using the calls reported by the TCGA consortium based on Affymetrix SNP 6.0 array data and the GISTIC 2.0 algorithm [10]. *PD-L1* status was assessed as "loss" for calls −2 and −1, as "normal" for call 0, and as "gain" for calls 1 and

2. Copy number calls and upper quartile normalized RSEM estimates of mRNA expression were obtained from the cBioPortal (http://www.cbioportal.org). Expression values were $\log2(x + 1)$ transformed prior to statistical analysis and visualization. Differential expression between tumors with and without *PD-L1* gains as well as between tumors with and without *PD-L1* losses was assessed using Welch's t-test. Hierarchical clustering was performed using Pearson correlations as similarity measure and the average linkage method to determine the distance between clusters. Non-significant fold changes were set to one before clustering. In a genome-wide mRNA expression analysis, a list of 75 strongly and recurrently up-regulated genes was identified by filtering for genes showing significant ($p < 0.05$) and at least 1.5-fold enhanced mRNA expression in tumors with *PD-L1* gains across at least 6 cancer types (Additional file 1: Table S1). The fold change threshold was added to the gene selection criterion to concentrate on strong expression changes that can be potentially therapeutically targeted. Pan-cancer significance was assessed by combining the *p*-values of specific cancer types using Fisher's method. Enrichment analysis of functional categories was performed based on gene ontology (GO) annotations obtained from msigdb v5.2 C5 (http://software.broadinstitute.org/gsea/msigdb) with significance assessment by Fisher's exact test (Additional file 2: Table S2). Among several enriched GO categories that were connected with the biological motives proliferation and immunology the largest of these, 'cell cycle' (GO:0007049) and 'immune system process' (GO:0002376), were utilized for annotation of the 75 genes. Statistics and graphics generation were performed using the programming language R. Everywhere, *p*-values < 0.05 were considered significant.

Results

Comparing tumors with and without *PD-L1* CNG in 22 cancer types, we found significant mRNA expression changes of *PD-L1* in 11 cancer types, of *PD-L2* in 9 cancer types and of *JAK2* in 15 cancer types (Table 1). Some of the detected *PD-L1* mRNA expression changes were very high such as in lung squamous cell cancer (fold change = 4.9), bladder cancer (fold change = 4.1), head and neck cancer (fold change = 2.9), cervical cancer (fold change = 2.7) and cutaneous melanoma (fold change = 2.2). Furthermore, we explored the global mRNA expression pattern of chromosome 9p using hierarchical clustering and heatmap visualization (Fig. 1). In the 11 cancer types with significant *PD-L1* mRNA expression changes, between 124 to 342 genes (15% to 42%) located on chromosome 9p were found to be differentially expressed. Comparing tumors with and without *PD-L1* loss, we identified 14 cancer types with significant *PD-L1* mRNA expression changes and 167 to 415 genes (20% to 51%) with modulated mRNA expression. Genes

Budczies *et al. BMC Medical Genomics* (2017) 10:74

Page 3 of 8

Table 1 Analysis of differential mRNA expression of *PD-L1*, *PD-L2* and *JAK2* in tumors with *PD-L1* CNG compared to tumors without *PD-L1* CNG

Cancer type	PD-L1		PD-L2		JAK2	
	fold change	p	fold change	p	fold change	*p*
Lusc	**4.9**	**1.60E-27**	**2.3**	**3.80E-13**	**1.8**	**3.70E-14**
Blca	**4.1**	**7.70E-14**	**2.6**	**2.80E-06**	**2**	**3.60E-14**
Hnsc	**2.9**	**1.30E-16**	**2.5**	**1.20E-14**	**2.1**	**7.10E-19**
cesc	**2.7**	**2.50E-08**	**2**	**9.00E-05**	**1.9**	**4.90E-07**
skcm	**2.2**	**0.0073**	1.5	0.12	**2**	**0.00026**
paad	2	0.14	1.6	0.5	1.3	0.23
sarc	**1.9**	**0.0028**	**2.2**	**0.00013**	**1.4**	**0.00042**
luad	**1.8**	**0.00078**	1.2	0.12	**1.4**	**7.60E-05**
prad	**1.8**	**9.00E-04**	**1.6**	**0.002**	**1.4**	**0.00017**
stad	**1.8**	**0.0026**	1	0.79	**1.4**	**0.0061**
kirc	1.7	0.18	1	0.98	1.2	0.15
ov	**1.7**	**5.20E-07**	**1.3**	**0.034**	**1.6**	**4.40E-12**
kirp	1.6	0.44	1.1	0.87	1.3	0.45
thca	1.5	0.45	1.4	0.3	1.2	0.4
gbm	1.4	0.34	1.6	0.1	**1.3**	**0.014**
ucec	1.4	0.17	1.2	0.51	1.2	0.28
brca	**1.3**	**0.016**	**1.3**	**0.002**	**1.3**	**2.40E-05**
lihc	1.2	0.52	-1	0.91	**1.5**	**0.0083**
coadread	1.1	0.62	1.1	0.59	**1.3**	**0.00038**
lgg	-1	0.88	**2.5**	**0.004**	**1.4**	**0.00027**
pcpg	−2.6	0.27	−1.3	0.13	−1.2	0.48

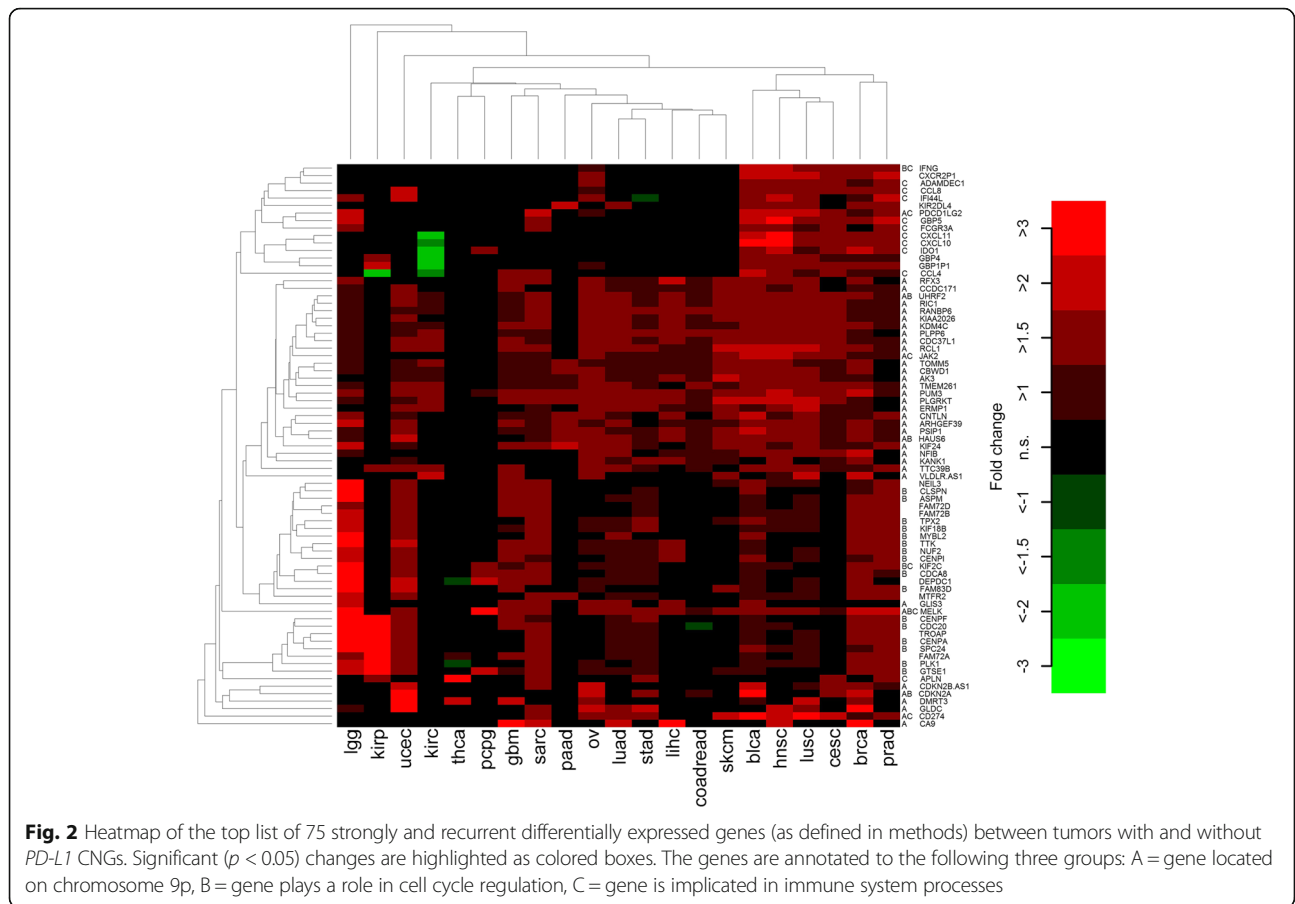Significant changes (*p* < 0.05) are highlighted

Investigated cancer types: Bladder Urothelial Carcinoma (BLCA), Breast invasive carcinoma (BRCA), Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), Colorectal adenocarcinoma (COADREAD), Glioblastoma multiforme (GBM), Head and Neck squamous cell carcinoma (HNSC), Kidney renal clear cell carcinoma (KIRC), Kidney Renal Papillary Cell Carcinoma (KIRP), Brain Lower Grade Glioma (LGG), Liver hepatocellular carcinoma (LIHC), Lung adenocarcinoma (LUAD), Lung squamous cell carcinoma (LUSC), Ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), Pheochromocytoma and Paraganglioma (PCPG), Prostate Adenocarcinoma (PRAD), Sarcoma (SARC), Skin Cutaneous Melanoma (SKCM), Stomach adenocarcinoma (STAD), Papillary Thyroid Carcinoma (THCA), and Uterine Corpus Endometrial Carcinoma (UCEC)

Bold data indicate significance (*p* < 0.05)

showing frequent up-regulation in tumors with *PD-L1* gains included *PD-L1, PD-L2, JAK2,* and *B4GALT1.* Genes showing frequent down-regulation in tumors with *PD-L1* losses included *PD-L1, PD-L2* and *IFNB1.*

Taking a more comprehensive approach, we analyzed the setting 'chromosome 9p gain' in a genome-wide context and identified 75 top genes that were strongly and recurrently up-regulated (Fig. 2 and Additional file 1: Table S1). In a pan-cancer analysis and after Bonferroni correction for 22,849 genes, 74 of these were significantly associated with 9p gain status. While 36 strongly up-regulated genes (48%) were located on chromosome 9p (including *PD-L1, PD-L2* and *JAK2*), the remaining 39 genes (52%) were located in other genomic regions. In particular, 21 genes were located in the core amplified region in chromosome 9p24 that we described in [8]. Twenty-two strongly up-regulated genes belonged to the GO category 'cell cycle' (*ASPM, CDC20, CDCA8,*

CDKN2A, CENPA, CENPF, CENPI, CLSPN, FAM83D, GTSE1, HAUS6, IFNG, KIF18B, KIF2C, MELK, MYBL2, NUF2, PLK1, SPC24, TPX2, TTK, UHRF2). The vast majority of these genes play a role in the coordinated regulation of microtubule assembly and chromosomal segregation during mitosis. Sixteen strongly up-regulated genes belonged to the GO category 'immune system process' (*ADAMDEC1, APLN, CCL4, CCL8, CXCL10, CXCL11, FCGR3A, GBP5, IDO1, IFI44L, IFNG, JAK2,* KIF2C, *MELK, PD-L1, PD-L2*). Most of these genes encode cytokines and chemokines but also link immune response with tumor metabolism, such as *IDO1* encoding indoleamine-2,3-dioxygenase 1. Enrichment of the both GO categories was highly significant (cell cycle: $p = 2.5E-10$, immune system process: $p = 0.00096$). Interestingly, as shown in Fig. 2, upregulation of immune system-related genes was particularly enriched in prostate adenocarcinoma, breast cancer, cervical cancer,

Fig. 1 Shaping of mRNA expression of chromosome 9p by copy number alterations in chromosome 9p including *PD-L1*. **a** Heatmap showing mRNA fold changes tumors with and without *PD-L1* gain. **b** Heatmap showing mRNA fold changes of tumors with and without *PD-L1* loss. Significant (*p* < 0.05) changes are highlighted as colored boxes. Genes belonging to recurrently amplified or deleted regions (as identified in [8]) are marked by a star

Budczies *et al. BMC Medical Genomics* (2017) 10:74

Page 5 of 8

Fig. 2 Heatmap of the top list of 75 strongly and recurrent differentially expressed genes (as defined in methods) between tumors with and without *PD-L1* CNGs. Significant (*p* < 0.05) changes are highlighted as colored boxes. The genes are annotated to the following three groups: A = gene located on chromosome 9p, B = gene plays a role in cell cycle regulation, C = gene is implicated in immune system processes

squamous cell lung cancer, head and neck cancer and bladder cancer, while genes regulating cellular proliferation were strongly expressed in glioma, papillary renal cell carcinoma and uterine corpus endometrial carcinoma well as in breast cancer, prostate cancer, sarcomas, and glioblastomas. Higher expression levels of genes on 9p were observed across most cancer types except for papillary thyroid carcinoma as well as pheochromocytoma and paraganglioma.

Discussion

Functional genomics analysis of *PD-L1* CNG tumors revealed strong and recurrent mRNA expression changes of genes within and outside chromosome 9p. Before, we showed that approximately half of the *PD-L1* CNGs in the major cancer types occur together with amplification of chromosome 9p or the entire chromosome 9 (non-focal gains), while the other half of *PD-L1* CNGs frequently co-occurs with gain of a 38-gene core amplified region located in chromosome 9p24 (focal gains) [8]. Here were analyzed focal and non-focal CNGs together and identified 75 genes (21 in located the core amplified region) that were strongly and recurrently up-regulated in *PD-L1* CNG tumors. The gene set was enriched for the biological

motives 'cell cycle' and 'immune system process' and included known drivers of tumor growth and drug targets.

A recent study showed that *PD-L1* CNG is a powerful predictive biomarker in patients with relapsed or refractory Hodgkin lymphoma who were treated with nivolumab [3]. In keeping with these data, we recently demonstrated that *PD-L1* amplification accompanied by overexpression can also be used to identify patients with solid tumors who benefit from *PD-1* blockade [4]. These data support a view that *PD-L1* CNGs could help to predict response to checkpoint inhibitors in solid tumors. Testing for *PD-L1* amplifications could be easily implemented in a routine setting using in situ hybridization based assays such as FISH or using targeted DNA sequencing [11]. However, prospective clinical trials and retrospective-prospective analyses of existing clinical trial tissue cohorts are warranted to get a comprehensive picture on the clinical utility of *PD-L1* CNGs in solid cancer types.

Copy number and gene expression analyses uncovered additional potential drug targets, such as amplified and overexpressed *JAK2* (Table 1), which has been implicated in the regulation of *PD-L1* expression [12] and appears to play a role in tumor growth and resistance to

Budczies *et al. BMC Medical Genomics* (2017) 10:74

Page 6 of 8

chemotherapy [13]. *B4GALT1* encoding β-1, 4-Galactosyltransferase is also frequently co-amplified and overexpressed, and has been demonstrated to mediate multidrug resistance in leukemia cell lines [14]. *PD-L2*, the gene next to *PD-L2* located about 40 kbp toward the centromere, has been demonstrated to be always co-amplified and co-deleted with *PD-L1* across major cancer types [8]. Consistent with this result, we here found the two genes to be co-regulated on transcriptional level in many cancer types. *PD-L2* is a second ligand for *PD-L1* inhibiting T cell activation [15] and has recently been demonstrated to be associated with clinical outcome in head and neck cancer independent of *PD-L1* [16].

Taking a broader view, our analysis identified two gene expression programs that were significantly associated with cancers harboring 9p gains: regulation of cell cycle and proliferation as well as modulating and fine tuning of immune cell response. As expected, the latter comprised *PD-L1*, *PD-L2,* and *JAK2* already discussed above. Additionally, however, we noted a specific set of genes encoding chemokines and cytokines, namely *CCL4*, *CCL8*, *CXCL10*, *CXCL11*, *IFI44L*, *IDO1*, and *IFNG* that were upregulated in 9p CNG cancers. Chemokines and cytokines are well known to guide macrophages, and T-cells including CD8-positive T-lymphocytes to the tumor microenvironment and are associated with outcome [17, 18]. As the RNA-seq data analyzed here were derived from the entire tumor including its stromal and inflammatory components we were unable to discern whether upregulated expression levels stem directly from tumor cells or adjacent stromal cells. However, these data indicate that cancers with 9p gains involving *PD-L1* are associated with a 'hot microenvironment' attracting effector cells of the immune system that could eliminate tumor cells as soon as checkpoint blockade is in place and counteracts the anergic state. On the other hand, chemokines are known to play a role in metastatic spread of tumor cells as they may express CC and CXC chemokine receptors [19–21]. Together with *PD-L1* CNG that hamper successful immune attack due to increased *PD-L1* protein levels, chemokine secretion could drive tumor survival by supporting local or distant spread. Similarly, while *IFNG* exerts clear anti-tumorigenic functions it appears to be also implicated in tumor progression in some settings [22, 23]. Interestingly, among the upregulated genes *IDO1* encoding indoleamine-2,3-dioxygenase 1 was identified. This enzyme, whose expression can be induced by *IFNG* [24], is centrally involved in the degradation of tryptophan in tumor cells whose depletion eventually results in cell cycle arrest and apoptosis of T-cells [25–28], thus possibly supporting *PD-L1* CNG tumors to escape immune surveillance. Conversely, this *IDO1*-mediated mechanism of immune suppression can be exploited therapeutically by

inhibition of the catalytic activity of *IDO1* [29] suggesting that dual inhibition of both *PD-1* and *IDO1* could be a promising approach to enhance efficacy of checkpoint blockade. Several studies investigating dual *IDO1-PD1* blockade in solid tumor types are underway and preliminary results are encouraging [30, 31].

The second expression program that we identified in cancers with 9p gains includes genes involved in formation of microtubules, chromosome segregation and governing mitosis. For example, *CDC20* encoding cell-division cycle protein 20 homologue plays a role in chromosome separation and transition to G1 phase after mitosis, and may be a suitable drug target [32, 33]. Similarly *PLK1*, which is well known to control metaphase to anaphase transition and mitotic exit, is upregulated in these cancers and pre-clinical as well as clinical data collectively suggest that *PLK1* inhibition can be a promising strategy in cancer therapy [34–36]. Recently, overexpression of *TTK*, another checkpoint in mitosis [37], was reported to occur in triple negative breast cancers [38], which frequently harbor *PD-L1* amplifications [2, 4]. In line with this finding, our analysis showed upregulation of *TTK* across many cancers that harbor CNG of 9p involving *PD-L1*. *MELK* encoding maternal embryonic leucine zipper kinase appears to play a role in governing proliferation and conferring anti-apoptotic properties to tumor cells. With the recent development of inhibitors [39], additional *MELK* inhibition may also be a therapeutic option in patients with *PD-L1* amplified cancers [40].

On a genome-wide scale, recurrent patterns of copy number alterations have been discovered across patients and cancer types suggesting their shaping by selective pressures and roles in tumor genesis and tumor growth [41, 42]. However, the effects of these, in particular of small gene dosage alterations including chromosome or chromosome arm gains or losses, on the phenotypic level are incompletely understood. In a recent pan-cancer analysis, a copy number alteration signature has been linked up-regulation of glycolysis [43], while chromosome 8p loss in breast cancer has been linked to alterations in lipid metabolism [44]. Here, we demonstrated a link of 9p gains to the regulation of proliferation and immune response. Our observations support a view of copy number alterations as hard-wired genetic changes that maintain cancer hallmarks including 'self-sufficiency in growth' and 'evasion from the immune system' from Hanahan's and Weinberg's list [45].

Conclusions

In summary, we identified a gene signature that is upregulated in *PD-L1* CNG tumors across many cancer types. The data contribute to understanding the biology of these cancers and highlight additional vulnerabilities that might be

Budczies *et al. BMC Medical Genomics* (2017) 10:74

Page 7 of 8

therapeutically exploitable thus possibly sustaining or enhancing efficacy of checkpoint blockers.

Additional files

Additional file 1: Table S1. Gene list of 75 top up-regulated genes identified in a genome-wide transcriptome analysis across 22 cancer types. The list was obtained by filtering for genes harboring significant and at least 1.5-fold enhanced mRNA expression in tumors with PD-L1 gains across at least 6 cancer types. Pan-cancer *p*-values were calculated using Fisher's method (* = significant, ** = significant after Bonferroni correction for 22,849 genes). The set of 75 genes was enriched for the chromosomal regions 9p13 to 9p24 (36 genes) as well as the biological processes 'cell cycle' (22 genes) and 'immune system process' (16 genes). (XLSX 34 kb)

Additional file 2: Table S2. Enrichment analysis of GO categories in the 75-gene list. (XLSX 240 kb)

Abbreviations
CNG: Copy number gain; JAK2: Janus kinase 2; PD – 1: Programmed cell death protein 1 (PDCD1); PD-L1: Programmed cell death 1 ligand 1 (CD274); PD-L2: Programmed cell death 1 ligand 2 (PDCD1LG2); TCGA: The Cancer Genome Atlas

Availability of data and materials
The data analyzed in this study are publicly available from the cBioPortal (http://www.cbioportal.org).

Authors' contributions
JB and AS designed the study, CD contributed to the study design. JB analyzed data and generated Figures and Tables. All authors contributed to the discussion. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Budczies et al. BMC Medical Genomics (2017) 10:74

Page 8 of 8

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