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Opinion

All is not lost: learning from 9p21 loss in cancer

Pavlina Spiliopoulou,[1,5] S.Y. Cindy Yang,[1,5] Jeff P. Bruce,[1] Ben X. Wang,[1] Hal K. Berman,[1,2] Trevor J. Pugh,[1,3,4] and Lillian L. Siu [1,*]

The cancer research community continues to search for additional biomarkers of response and resistance to immune checkpoint treatment (ICT). The ultimate goal is to direct the use of ICT in patients whose tumors are most likely to benefit to achieve a refinement that is equivalent to that of a genotype-matched targeted treatment. Dissecting the mechanisms of ICT resistance can help us characterize ICT nonresponders more efficiently. In this opinion, we argue that there may be additional knowledge gained about immune evasion in cancer by analyzing the loss of the human 9p21.3 locus; as an example, we highlight findings of 9p21.3 loss from the investigator-initiated, pan-cancer INSPIRE study, in which patients were treated with pembrolizumab (anti-PD-1 antibody) ICT.

Highlights

Human chromosomal 9p21.3 loss is emerging as a biomarker of a 'cold' tumor immune microenvironment and poor response to cancer immunotherapy with ICT.

9p21.3 loss might exert its effect on immune evasion through cell cycle, metabolic, and/or type I interferon (IFN) response pathways. It might also be implicated in long-distance transcriptional regulation through the presence of an abundance of DNA regulatory elements.

We posit that, by including 9p21.3 copy number loss into response score tools, alongside PD-L1 expression and tumor mutational burden, it may be possible to better define those patients who would not derive benefit from ICT.

Defining '9p21-ness' with further mechanistic research might help us identify strategies for ICT enhancement and novel pharmacological vulnerabilities.

9p21.3: a locus of interest in solid cancers

Fifteen percent of all human cancers demonstrate homozygous deletion of the chromosome 9p21 locus [1]. A recent study by Han and colleagues shed new light on the 21.3 locus of the 9p chromosome arm (henceforth referred to as 9p.21) [2]. By interrogating large-scale, pan-cancer genomic data from The Cancer Genome Atlas (TCGA), the authors showed across multiple cancer types that 9p21 loss acts as a marker of poor prognosis, a finding that was previously observed only for individual tumor types, such as renal cell, esophageal, prostate, and breast cancers [3–11]. Their exploration demonstrated a clear association between homozygous loss of 9p21 and shorter overall survival. Although not as strong an effect, loss of heterozygosity (LOH) for 9p21 was also associated with worse prognosis [2]. Importantly, the authors demonstrated that tumors have a differential response to **ICT** (see Glossary), depending on their 9p21 somatic copy number alteration (SCNA) status. Using the **Response Evaluation Criteria in Solid Tumors** (RECIST v1.1) framework to evaluate objective responses, patients with cancer with 9p21 loss exhibited attenuated responses to ICT, and shorter progression-free survival and disease-specific survival compared to patients with wild-type 9p21 [2].

To elucidate possible mechanisms underpinning these associations, Han and colleagues conducted an immunogenomic analysis of bulk RNA-sequencing (RNA-seq) data of the TCGA pan-cancer cohort. This revealed that tumors that have lost the 9p21 locus are characterized by a **'cold' immune microenvironment**, with reduced expression of immunostimulatory genes, such as *CXCL9*, *CXCL13*, *CCL5*, *CD27*, and *ICOS*; and parallel upregulation of immunosuppressive mediators, such as *CD155*, *TGFB1*, *CD73*, *VEGFA*, and *CD276*. The shift in these immune gene networks indicates that tumors with 9p21 loss could foster a tumor microenvironment (TME) that supports immune evasion. The authors also observed in tumors with 9p21 loss compared to tumors with intact 9p21: reduced abundance of tumor-infiltrating lymphocytes (TILs) by immune deconvolution analysis of bulk RNA-seq data, including $CD8^+$ cytotoxic T lymphocytes, B lymphocytes, and natural killer (NK) cells; diminished **programmed cell death ligand 1 (**PD-L1) expression; as well as decreased T cell receptor (TCR) richness and diversity [2]. This suggested that 9p21 locus deficiency leads to an immunosuppressive environment

[1]Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
[2]Toronto General Hospital, University Health Network, Toronto, ON, Canada
[3]Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada
[4]Ontario Institute for Cancer Research, Toronto, ON, Canada
[5]These authors contributed equally to this work.

*Correspondence: lillian.siu@uhn.ca (L.L. Siu).

hindering tumor responses to ICT and, therefore, that 9p21 status could be a predictive marker of ICT resistance.

Han and colleagues further investigated the effect of 9p21 status on predicting ICT resistance and reported that its predictive value was stronger than that of well-established, biology-driven factors, such as **tumor mutational burden** (TMB) or PD-L1 expression, both individually or combined [2]. Moreover, a **response score** of all three variables (9p21, TMB, and PD-L1) could predict treatment response and patient survival with higher statistical confidence compared to TMB and PD-L1 tested individually [2]. This motivated us to examine this framework in a previously reported, investigator-initiated Phase II clinical trial of the ICT, **pembrolizumab**, in patients with advanced cancers (Investigator-initiated Phase-2 Study of Pembrolizumab Immunological Response Evaluation; INSPIRE; NCT02644369[i]). Based on such studies, we argue that, by assessing 9p21 loss in solid tumors for patients receiving ICT, we can gather valuable information and knowledge about mechanisms of immune evasion in cancer.

9p21.3 as a tumor suppressor beyond CDKN2A and MTAP

In the study by Han *et al.* [2], the authors defined '9p21 loss' mainly by perturbations in the tumor suppressor genes encoding cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and *S*-methyl-5'-thioadenosine phosphorylase (*MTAP*). The latter resides 100 kb further away from *CDKN2A* and is co-deleted in 80–90% of cancers with *CDKN2A* deletion [12]. *CDKN2A* encodes the $p14^{ARF}$ and $p16^{INK4A}$ proteins, which signal through the p53 and retinoblastoma (Rb) protein pathways, respectively [13]. On the one hand, *CDKN2B,* arranged in tandem with *CDKN2A,* encodes $p15^{INK4B}$, a cell cycle regulator that exhibits inhibitory activities against cyclin-dependent kinases CDK4 and CDK6 [14], with resultant CDK4/6 inhibition that blocks the G1–S-phase cell cycle transition [14–16]. On the other hand, MTAP is a key enzyme in the salvage pathway of methionine; it metabolizes the by-product of polyamine synthesis, 5 -methylthioadenosine (MTA), leading to the final step of methionine and adenine regeneration [17].

The link between the 9p chromosome arm and cancer was discovered more than 30 years ago [18], but a plethora of recent research outputs is revalidating this connection. One study highlighted a region of ~700 kb centromeric of *CDKN2A* that demonstrated frequent homozygous deletions in more than 600 human cancer cell lines [19]; however, larger deletions (up to 9.2 Mb) were also observed. The 9p21 region harbors a cluster of interferon (IFN) genes [20] and other genes with less well-studied roles in cancer, including *ELAVL2*, *TUSC1*, *LINGO2*, and others [21]. Genome-wide association studies (GWAS) have also uncovered SNPs in the 9p21 locus associated with cancer susceptibility [21]. While most risk-associated SNPs cluster on *CDKN2A/CDKN2B/ANRIL* and *MTAP* [22–29], SNPs residing in other genes of interest within the 9p21 locus have also been uncovered. For instance, SNPs in the gene encoding IFN omega 1 (*IFNW1)* have been implicated in the pathogenesis of renal cell and breast cancers [21]; SNPs in the gene encoding ELAV-like RNA Binding Protein 2 gene (*ELAVL2)* were found in patients with lung and breast cancers [21]. Germline perturbations affecting the 9p21 region have also been discovered in individuals with certain familial cancers, such as melanoma. Notably, two recently reported cases in familial syndromes manifesting with melanoma, and neural system and breast cancers, led to the discovery of deletions that span and extend further than the *CDKN2A/B* region, affecting up to 25 genes [30,31].

Many researchers primarily focused on *CDKN2A/CDKN2B* and *MTAP* as putative drivers of the effect of 9p21 loss on cancer progression and patient survival, in large part due to their well-described roles in cell cycle regulation and tumor metabolism [22,32–35]. However, others have evaluated the integrity of the 9p21 locus in its entirety, using methods such as fluorescence

Glossary

cGAS–STING cytosolic DNA-sensing pathway: intracellular process to detect cytosolic DNA (microbial or of self-origins) leading to activation of type I IFN responses and NF-κB pathways.
CIBERSORT: bioinformatics algorithm that provides quantitative estimates of immune cell populations within a tumor sample from its bulk RNA-seq profile.
'Cold' immune microenvironment: classification of the TME based on the abundance and distribution of infiltrating lymphocytes. A 'cold' designation describes the absence or low abundance of lymphocyte infiltration within the tumor tissue. ICT has limited clinical efficacy in tumors with a 'cold' immune microenvironment.
Enhancers: DNA regulatory sequences that, when bound by transcription factors, enhance transcription of an associated gene.
Immune checkpoint treatment (ICT): therapeutic strategy inhibiting immune checkpoint pathways. These pathways are co-opted by cancer as an immune evasion mechanism. Examples of US FDA-approved ICTs in cancer include monoclonal antibodies targeting CTLA-4, PD-1, and PD-L1 immune checkpoint receptors and ligands.
Microsatellite status: marker describing the level of mutation rate found in repetitive DNA sequences called microsatellites; reflects the integrity (or not) of DNA mismatch repair mechanisms. Tumors with a defective DNA mismatch repair system and mutated microsatellites are known to have 'microsatellite instability'.
Pembrolizumab: humanized IgG4 monoclonal antibody targeting the PD-1 immune checkpoint receptor used to treat many cancer types. First FDA-approved tissue-agnostic cancer drug for the treatment of metastatic or unresectable solid tumor with DNA mismatch repair deficiency or microsatellite instability.
Programmed Cell Death Ligand 1 (PD-L1): immune checkpoint ligand expressed on the cell surface of tumors, immune cells, and other cell types. ICT may be more effective in tumors with high cell surface expression of PD-L1 detectable by immunohistochemistry.
Response Evaluation Criteria in Solid Tumors (RECIST): set of clinical and/or radiological criteria that provide a methodology to evaluate the activity and

in situ hybridization (FISH) or SCNA detection through next-generation sequencing [3,5,8,21,36–38]. For instance, 16% of Epstein–Barr virus (EBV)-driven nasopharyngeal carcinoma cases have been reported to include homozygous deletion of the 9p21 region, including loss of the upstream type I IFN genes [39].

These emerging data suggest that, notwithstanding the significant roles of *CDKN2A/CDKN2B* and *MTAP,* there may be other essential mediators of oncogenicity in this locus.

9p21.3 loss in the INSPIRE clinical study

Intrigued by the findings of Han *and colleagues* [2], and to highlight an example of the significance of 9p21 loss as a candidate predictive marker in cancer, we performed an independent validation of somatic 9p21 SCNA status as a biomarker of unfavorable clinical outcomes with ICT. Specifically, we used clinical and molecular data sets collected from INSPIRE [40,41]. INSPIRE was a nonrandomized, single-arm, single-institution study of pembrolizumab in patients with advanced solid tumors allocated into five cohorts: squamous cell cancer of head and neck (HNSCC), triple-negative breast cancer (TNBC), high-grade serous ovarian carcinoma (HGSC), malignant melanoma (MM), and mixed/rare solid tumors (RST). The study aimed to evaluate the genomic and immune landscapes of peripheral blood and tumors following pembrolizumab treatment and has completed recruitment. For our current analysis, we stratified patients into four groups as per 9p21 SCNA status from tumors collected before pembrolizumab treatment: gain, wild type, loss, and homozygous deletion.

Tumor 9p21 SCNA status in 152 tumors from 99 patients was inferred from the segmented log2-transformed depth-ratio of the 9p21.3 chromosome region (hg38 chr9: 19.9–25.6 Mb), captured by exome sequencing. The inference was conducted by two independent reviewers (22/152; 14% discordant calls resolved by joint review) (Figure 1A). The 9p21 SCNA status for each patient was assigned based on the presence of SCNA seen in either formalin-fixed paraffin-embedded archival tumor or fresh-frozen tumor biopsy collected at baseline before treatment. Deep deletion, likely homozygous deletion (of two copies), was observed in tumors from 5% (5/99) of patients; one-third (38%; 38/99) of patients had a single DNA copy loss (log2R <0); 44% (44/99) with no change (log2R = 0); and 12% (12/99) with copy number gain (log2R >0) (Figure 1B). In keeping with the analysis conducted in [2], we used matched RNA-seq data to confirm that tumors with 9p21 loss had reduced expression of two tumor suppressor genes located within the 9p21 locus, *MTAP* (P = 0.008, two-sided Wilcoxon rank sum test) and *CDKN2A* (P = 0.01, two-sided Wilcoxon rank sum test), compared to tumors with intact 9p21 (Figure 1C). While the cancer cohort composition was similar when compared between 9p21-loss and wild-type groups (Figure 1D), three out of five tumors with homozygous deletion loss belonged to the HNSCC cohort. Our SCNA analysis was a small-scale, pan-cancer analysis, akin to that by Han *et al* [2]. Our analysis revealed a lower rate of 9p21 homozygous deletions than that observed in [2]. We corroborated the genomic loss of 9p21q with diminished gene expression amounts of *MTAP* and *CDKN2A*. This evaluation gave us confidence to proceed with our survival and response analysis.

We confirmed the 9p21-loss SCNA status as an independent genomic biomarker associated with decreased overall survival during pembrolizumab treatment, with statistical significance (P = 0.005, Figure 2A). This association retained significant effects (P = 0.025; adjusted Hazard Ratio, 2.17; 95% CI, 1.11–4.64) when incorporated into a combined model including tumor PD-L1 immunohistochemistry (IHC) score and TMB (Figure 2C). While the association in progression-free survival (PFS) was modest, we observed trends consistent with shorter PFS in patients with 9p21-loss compared to wild type (P = 0.07, Figure 2B,D). Furthermore, we confirmed a lower clinical benefit

efficacy of cancer therapeutics in solid tumors, by assessing changes in tumor burden.

Response score: novel predictive biomarker developed by Han and colleagues [2] derived from a composite of somatic 9p21-loss status, tumor mutation burden, and PD-L1 protein expression on immune cells.

Tumor mutation burden (TMB): measurement or estimation of the total number of somatic single nucleotide variants within the tumor genome (typically expressed in units of absolute number of variants or the frequency of variants across the genome); a predictive biomarker of immune checkpoint therapy.

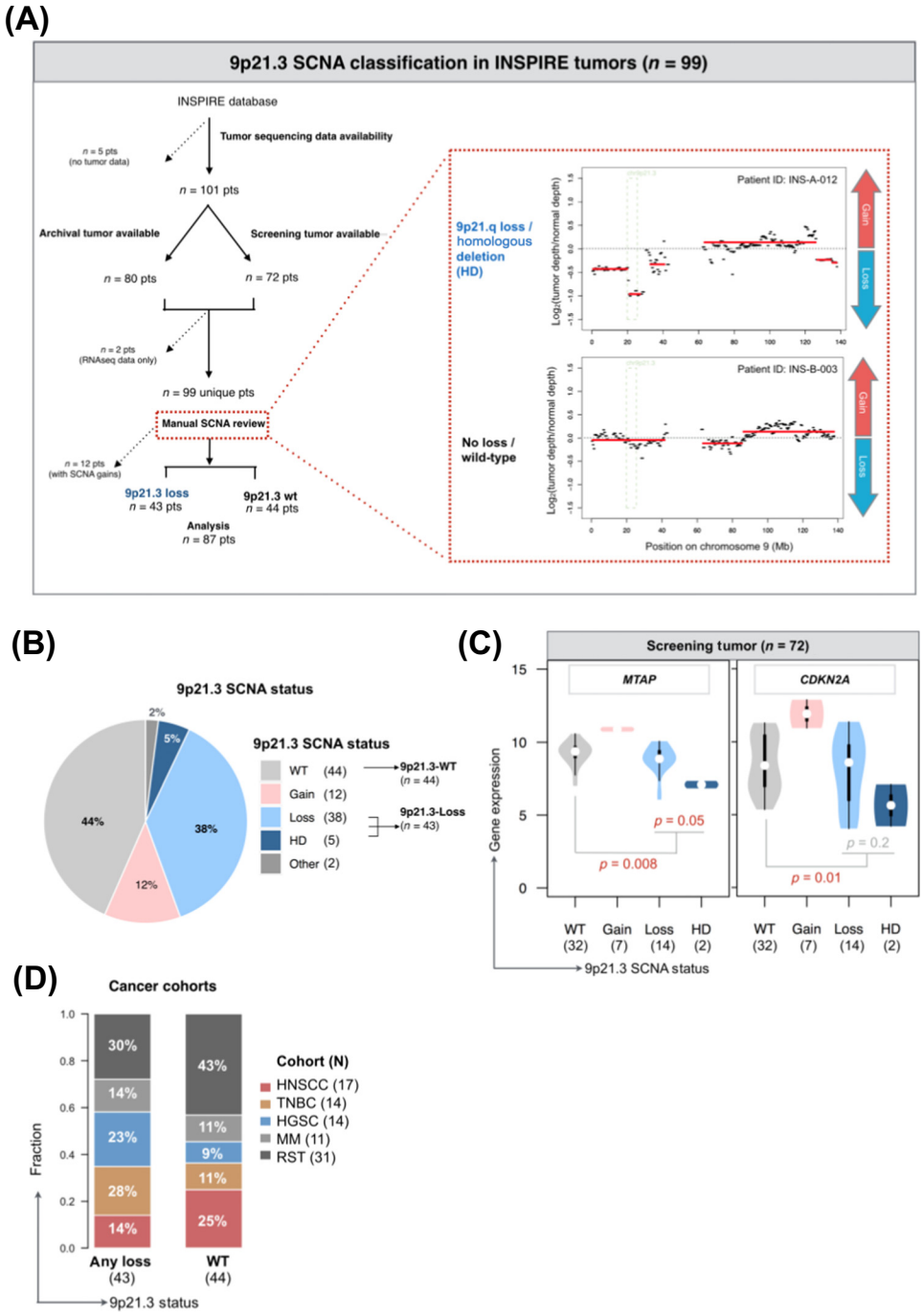

Figure 1. 9p21.3 copy number alteration (CNA) loss in solid tumors before anti-programmed cell death 1 (PD-1) antibody treatment. (A) Consort diagram and examples of chromosome 9 $\log_2$depth ratio from paired tumor/normal exome sequencing illustrating 9p21.3 loss (INS-A-012) and wild type (WT; INS-B-003). (B) Distribution of 9p21.3 somatic CNA (SCNA) status in the INSPIRE data set [40,41]. (C) Comparison of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and *S*-methyl-5′-thioadenosine phosphorylase (*MTAP*) transcript abundance by RNA-sequencing between 9p21.3 SCNA groups. (D) Distribution of solid tumor types with 9p21.3 loss and WT SCNA status within the INSPIRE data set. *P*-values and statistical

(Figure legend continued at the bottom of the next page.)

rate in the 9p21-loss group compared to wild type (14% versus 39%, $P = 0.02$, two-sided Fisher's exact test) (Figure 2E). A similar trend was noted for objective response (15% versus 25%, $P = 0.28$, two-sided Fisher's exact test) (Figure 2F). These results support somatic 9p21 copy number loss as a candidate genomic biomarker of resistance to ICT, coupled with shorter survival outcomes, for these tumor types.

9p21.3: an emerging role in sculpting the tumor microenvironment

While the role of 9p21 in tumorigenicity is obvious given that the locus harbors major cell cycle mediators, the link between 9p21 and the TME is only recently starting to emerge. Wells and Morawski previously reviewed the mechanisms through which *CDKN2A* and the cyclin-dependent kinase (CDK) cascades can program T cell biology in a T cell intrinsic manner [42]. However, how does the CDKN2A–CDK axis modulate the TME?

Tumors from patients with melanoma harboring intact *CDKN2A* have denser $CD8^+$ T cell infiltration and demonstrate enriched T cell-recruiting chemokine networks, as evidenced by RNA-seq data analysis from clinical samples and additional correlation with IHC staining [43,44]. Furthermore, in a study in which murine *Cdkn2a* was re-expressed by lentiviral infection in the B16-F10 melanoma cell line ($Cdkn2a^{-/-}$), the transcriptional output of chemokines, such as *Ccl4*, *Ccl5*, *Cxcl9*, *Cxcl10*, and *Cxcl11*, increased relative to the $Cdkn2a^{-/-}$ control B16-F10 cell line [43]. After uncovering a gene-expression signature deemed to predict intrinsic ICT resistance in patients with melanoma, others showed that, when mimicking the function of *CDKN2A*-encoded $p16^{INK4A}$ protein by pharmacologically inhibiting CDK4/6 (CDK4/6i), the ICT-resistant milieu could be repressed in the B16 melanoma mouse model [45]. Specifically, when C57BL/6 mice bearing ICT-resistant B16 tumors that express the aforementioned gene-expression signature were treated with the combination of CDK4/6i and ICT, survival was longer than for those treated with either CDK4/6i or ICT alone. Moreover, this was associated with a reduction in tumor volume [45]. Together, these findings are supported by data from 10 000 pan-cancer tumor samples showing that high *CDKN2A* expression can correlate with increased intratumoral infiltration by activated $CD4^+$ and $CD8^+$ T cells, as well as NK cells [46]. By contrast, *CDKN2A* loss of function in nonsmall cell lung cancer and urothelial cancer can predict resistance to ICT, shorter survival, and low amounts of intratumoral immune infiltrates [47,48]. Based on these findings, one might surmise that the effect of 9p21 loss on the tumor immune phenotype is partly *CDKN2A/B* driven, although this warrants robust investigation.

The positioning of type I IFN genes on 9p21 could not be more intriguing and, yet, their deletion as a result of 9p21 loss remains understudied. Previous work revealed that patients with melanoma lived longer when the gene networks of their tumors clustered within a type I IFN-stimulated gene-high ('ISG-high') signature. Conversely, tumors with an 'ISG-low' signature demonstrated SCNA loss of 9p21, with deletions that not only affected *CDKN2A/CDKN2B*, but also extended bi-directionally and included the IFN cluster [49]. Recently, large data-set interrogation demonstrated that deletion of the type I IFN gene cluster correlated with decreased overall survival in patients with uterine, renal, and brain cancers [50].

In addition to the CDKN2A axis and inflammatory mediators, such as type I IFN described above, reprogramming of the tumor stroma might also be achieved through metabolic network vulnerabilities that exist secondary to MTAP deficiency. For instance, in cancer cell lines, MTAP

significance as evaluated by two-sided Wilcoxon rank-sum tests. Abbreviations: HD, homologous deletion; HNSCC, head and neck squamous cell carcinoma; HGSC, high-grade serous ovarian cancer; MM, metastatic melanoma; RST, rare solid tumors; TNBC, triple-negative breast cancer. Statistically significant *P*-values ($P < 0.05$) are shown in red.

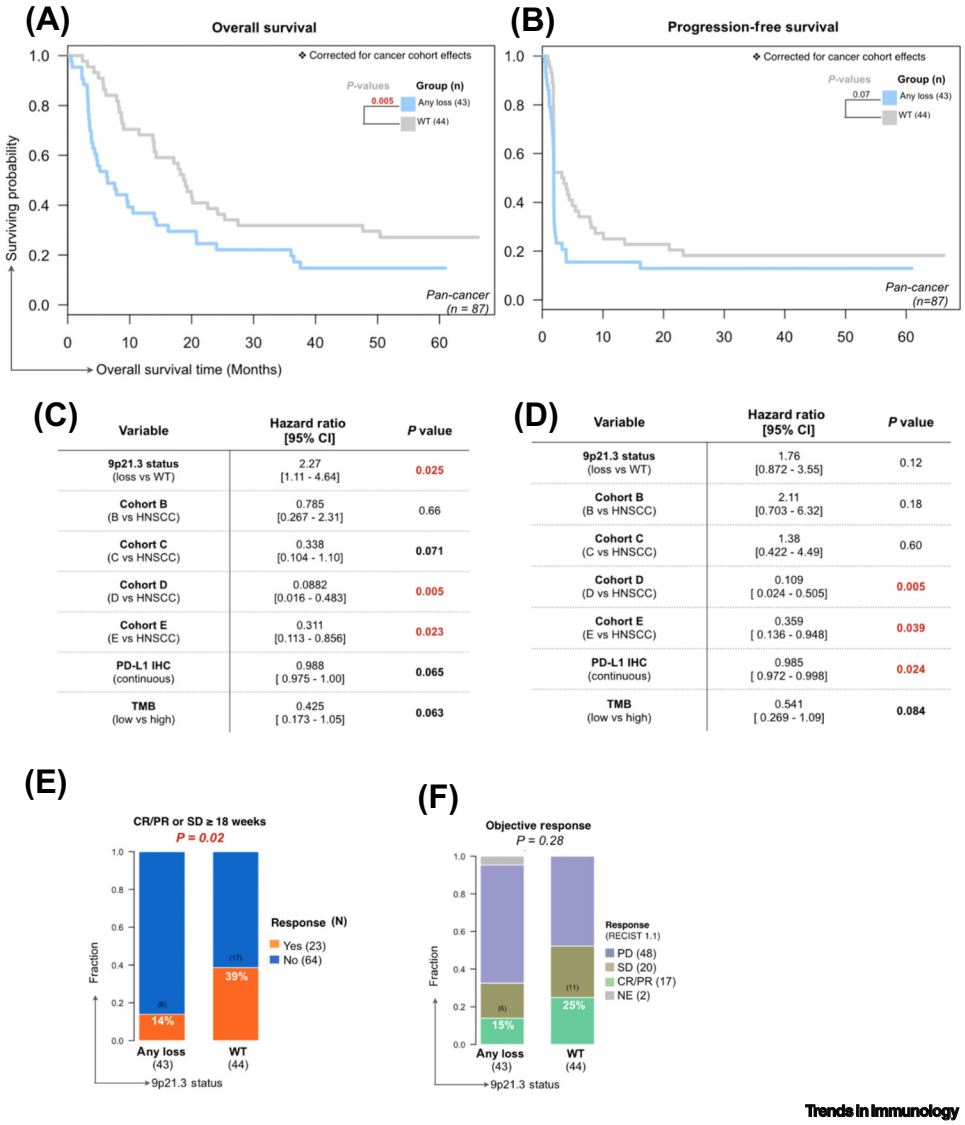

Figure 2. Validation of 9p21.3 somatic copy number alteration (SCNA) loss as a putative biomarker of poor clinical outcome to anti-programmed cell death 1 (PD-1) antibody treatment in solid tumors. Comparison of Kaplan–Meier estimates of (A) overall survival (OS) and (B) progression-free survival (PFS) between 9p21.3 SCNA groups (any loss versus wild-type; WT) with corrections for cancer cohorts. (C) Tables of results from a multivariable Cox-model analysis including 9p21.3 status, cancer cohorts, PD-L1 immunohistochemistry, and tumor mutation burden (TMB) for (C) OS and (D) PFS. (E) Comparison of clinical benefit rate to pembrolizumab [overall response of complete response (CR), partial response (PR), or stable disease (SD) for more than 18 weeks] between 9p21.3 loss and WT patients. (F) Comparison of objective response to pembrolizumab (overall response of CR or PR) between 9p21.3 loss and WT patients. For (E,F), *P*-values and statistical significance were evaluated by two-sided Fisher's exact tests comparing the proportion of responses (clinical benefit and objective response) between 9p21.3 SCNA groups. Abbreviations: HD, homologous deletion; PD, progressive disease; PD-L1, programmed cell death ligand 1; RECIST 1.1, Response Evaluation Criteria in Solid Tumors 1.1. Cohort A, squamous cell cancer of the head and neck; Cohort B, triple-negative breast cancer; Cohort C, high-grade serous ovarian cancer; Cohort D, malignant melanoma and Cohort E, mixed/rare solid tumors. Statistically significant *P*-values *(P < 0.05)* are shown in red.

deficiency leads to accumulation of the metabolite MTA [51,52] and abundant MTA intracellularly results in modification of several signaling pathways, including inhibition of protein arginine methyltransferase 5 (PRMT5) activity [53–56]. This phenomenon has been observed in a variety of

human cancer cells lines, such as melanoma, pancreatic, colorectal, and breast cancers. In turn, PRMT5 is a methyltransferase with multiple roles in key protein methylation and, therefore, can modulate the gene expression of many biosynthetic pathways [53,57,58], including proliferation networks in lung cancer, for example [59]. The interplay between metabolomics and methyltransferase activity is demonstrated in preclinical lung cancer models, in which arginine methyltransferase deficiency can drive $CD8^+$ T cell transition to terminal differentiation and impaired antitumor immune responses [60]. Further experiments have confirmed that tumor-derived MTA impedes antigen-specific cytotoxic $CD8^+$ T lymphocyte activation, possibly mediated through inhibition of arginine methylation of STAT1, a crucial activator of the IFN response [61,62]. In addition, interrogation of human tumors revealed that MTA accumulation could drive T cell dysfunction in hepatocellular carcinoma, whereas in glioblastoma, it drove repolarization of macrophages into a protumor, anti-inflammatory phenotype [63,64].

In sum, the effects of 9p21 loss on TME modulation appear to be multifactorial: cell cycle, IFN pathways, and metabolic regulators of immunity might all be important mediators in reinforcing conditions of immunosuppression.

To expand on the observation that tumors with 9p21 loss may be immunologically 'cold' [2], we sought to delineate the tumor immunological status in the INSPIRE study data set [65] for illustration purposes. We evaluated tumor PD-L1 positivity by IHC (Figure 3A) ($P = 0.30$, two-sided Fisher's exact test) as well as TIL composition via **CIBERSORT** [66,80] deconvolution from bulk tumor transcriptome profiles (Figure 3B). No notable ($P > 0.05$, Wilcoxon-rank sum tests) differences were observed in any of these measures in tumors with and without 9p21 loss. However, when the TIL content was interrogated via Hematoxylin and Eosin (H&E) staining (data previously presented in [65]), we detected a trend toward a greater intratumoral TIL score (TIL = 3) in patients with wild-type 9p21 compared to those with 9p21 loss (28% versus 10%, $P = 0.052$, exact Fisher's test; Figure 3C). Representative H&E-stained slides are shown in Figure 3D. The discrepancy between immune cell type inference from RNA-seq and histological results could be explained, in part, by the lack of spatial information and gene-expression signal dilution when assessing the TME through bulk tumor tissue sequencing. Nevertheless, the analysis from the INSPIRE study data set corroborates data from Han *et al.* [2], suggesting that the underlying mechanisms via which 9p21 loss predicts ICT resistance could be immune mediated.

Of note, a recent report indicated that 9p21 deletion correlates with worse survival during ICT in patients with metastatic renal cell carcinoma (mRCC), whereas it has no effect on survival of patients with mRCC treated with mTOR inhibitors [67]. In contrast to the INSPIRE analysis described above, RCC tumors that were enriched for 9p21 chromosomal loss were not 'cold', but were instead infiltrated by $CD8^+$ T cells [67]. RCC has always challenged the conventional paradigm that immune infiltration predicts better prognosis [68,69] so this finding is perhaps not as surprising, and it certainly does not weaken the role of 9p21 loss as a predictor of ICT resistance; instead, it creates an intriguing hypothesis that there may be alternative pathways involved in such ICT resistance.

Others have investigated the potential effect of 9p21 loss in renal tissues specifically. In immortal human kidney epithelial cells (HEK), short hairpin (sh)RNA-mediated *CDKN2A/CDKN2B* suppression did not increase cell growth potential, whereas CRISPR/Cas9-mediated deletion of the entire 9p21 locus increased both cell growth and colony formation [33]. Using chromatin immunoprecipitation sequencing (ChIP-Seq), one study showed that, in HEK cells with or without *CDKN2A/B* knockdown, 9p21 deletion decreased methylation of the promoter region of the transcription factor Homeobox protein, *HOXB13* [33]. This consequently activated *HOXB13*

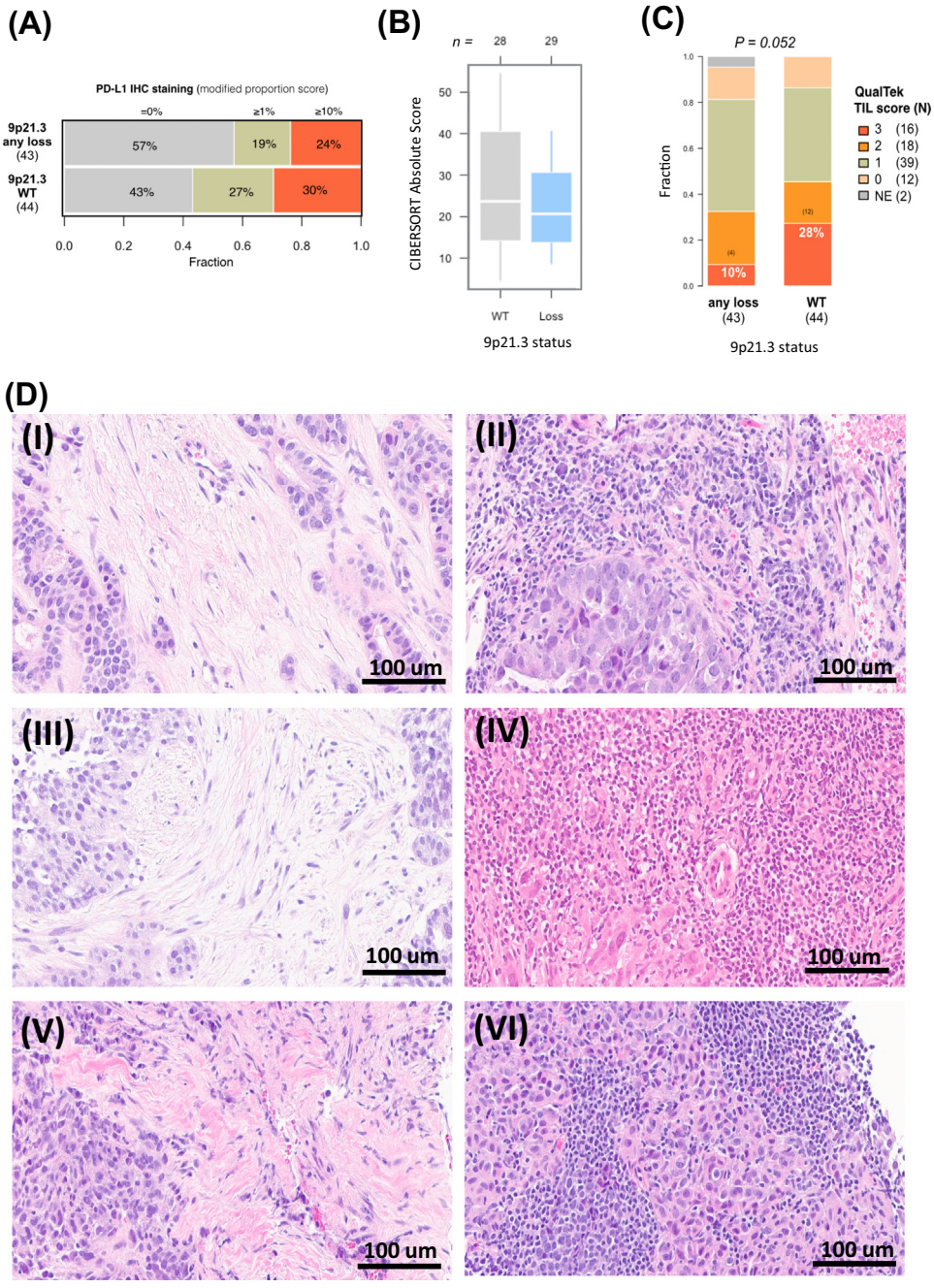

Figure 3. Tumor immune microenvironment differences between tumors with wild-type (WT) 9p21.3 and 9p21.3 loss. (A) Distribution of baseline tumor programmed cell death ligand 1 (PD-L1) immunohistochemistry (IHC) scores in 9p21.3 loss versus 9p21.3 WT patients. *P*-values were calculated using two-sided Fisher's exact test. (B) Comparison of CIBERSORT [66,80] absolute score [estimates total tumor-infiltrating lymphocytes (TIL) abundance] between WT and 9p21.3 loss in tumors at baseline. (C) Comparison of TIL scores (0–3) from Hemolysin and Eosin (H&E)-stained micrographs of WT and 9p21.3 loss in baseline tumors. *P*-values were calculated using two-sided Fisher's exact test comparing proportion of high TIL score (TIL score = 3) samples between 9p21.3 loss and WT groups. (D) Representative H&E micrographs of intratumoral TILs comparing carcinomas with 9p21 loss (images in left column: I,

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expression (encoding a major transcriptional regulator of development and differentiation [70]), which, in turn, facilitated tumor growth *in vitro* [33]. Therefore, this observation suggested that 9p21 has a broad transcriptional effect on multiple pathways; hence, its loss might lead to the perturbation of different processes contributing to the 9p21 phenotype, at least in RCC.

New mechanistic explanations of 9p21 tumorigenesis are gradually emerging, raising the question of whether there are wider genomic interactions triggered by 9p21 loss that have not yet been unveiled and which might impact tumorigenesis, progression, and the modulation of the tumor immune microenvironment.

9p21.3 somatic copy number alterations as a form of chromosomal instability in cancer

SCNAs are widespread in tumors and have emerged as significant drivers of immune-evading tumor phenotypes [1,71]. As a form of chromosomal instability, segmental (such as SCNAs) or whole-chromosome aneuploidies can lead to DNA spilling into the cytosol [72]. Subsequent activation of the **cGAS–STING cytosolic DNA-sensing pathway** and downstream noncanonical NF-κB signaling can contribute to, or promote, metastasis [72]. Indeed, a variety of chromosomal-level imbalances were found to inversely correlate with leucocyte infiltrates in a pan-cancer manner in a TCGA analysis of more than 10 000 genomes [73], with the correlation being strongest in pancreatic adenocarcinoma and HNSCC. Another report indicated that, in a cohort of patients with melanoma treated with ICT, somatic 9p arm loss was frequent among patients whose disease did not respond to either anti-PD-1 or anti-CTLA-4 antibody treatments [74]. Moreover, chromosomal 9p loss was associated with reduced expression of genes important for immune-related pathways, such as those involved in 'immune response', 'defense response', and 'leucocyte activation', among others, as evidenced by Gene Set Enrichment Analysis annotation, following RNA-seq interrogation. This would provide a credible explanation to support the observed poor response to ICT.

A differing mechanistic pathway between whole-chromosomal/chromosomal arm alterations and focal SCNA (e.g., loss of the 9p21 locus) has been investigated. One study collated results from large-scale data for SCNAs (SNP-array based data), point mutations, RNA-seq, microarray expression data, and clinical parameters from TCGA ,and correlated those with established immune gene signatures [75]. The results showed that immune-evasion tumor phenotypes could be better predicted by larger forms of aneuploidy, such as arm-level or chromosomal-level alterations, whereas focal SCNAs were mainly exerting their effects through the action of specific genes targeted by these SCNAs. Although these findings do not support a direct mechanistic role of 9p21 SCNA as an autonomous initiator of immune evasion, one should not dismiss the importance of the 9p21 locus as a putative transcriptional regulator. 9p21 is abundant in transcription regulatory elements, such as **enhancers**, and it corresponds to the second most-dense region of regulatory elements in the genome [76]. Of note, a report showed that enhancer elements on 9p21 could regulate the expression of all *CDKN2A/B*, *MTAP*, and *IFNA21* loci, and in turn, be modulated by IFNγ signaling, in the context of coronary artery disease, as evidenced by the integration of transcriptomic, RNA-seq, and ChIP-seq data [76]. This suggested that 9p21 is involved in spatial interactions that could result in long-distance modulation of gene transcription, a hypothesis that merits future investigation. Moreover, we

triple-negative breast cancer (TNBC); III, high-grade serous ovarian carcinoma (HGSOC); V, melanoma) versus carcinomas WT for 9p21.3 (images in right column: II, TNBC; IV, HGSC; and VI, melanoma). Images are digitized at 20× magnification. Abbreviations: NE, non-evaluable, PD-1, Programmed cell death 1; SCNA, somatic copy number alteration. Nonsignificant *P*-values are not shown.

posit that such interactions might shed more light on immune response mechanisms that are regulated by 9p21 loss.

Although not supported by robust evidence to date, 9p21 loss, as a form of chromosomal instability, still merits further investigation for a potential immunosuppressive role.

Concluding remarks

In 9p21 loss in cancer, focus was drawn for years to the *CDKN2A/CDKN2B* cluster, owing to its robust tumor suppressor properties, with the remainder of the 9p21 locus having been relatively understudied. Investigations into the role of type I IFN genes and metabolic polyamine pathways have recently emerged to show that, at least in part, mechanisms other than *CDKN2A/CDKN2B* modulate interactions that lead to an immunosuppressive microenvironment. In addition, an interesting example of the putative transcriptional regulation of 9p21 suggested that genomically distant interactions, through enhancer elements on 9p21, could control the output of key transcription factors (such as HOXB13) and might alter tumor responses. These observations are being investigated as potential drivers shaping the TME in a variety of cancers.

Findings on the role of 9p21 in shaping an immune phenotype that is not amenable to rescue by ICT are extremely topical, because the importance of identifying nonresponders to ICT cannot be overstated. The wide application of ICT makes it imperative that clinicians are able to use a robust marker that can help predict clinical responses (or at least be complementary to other measures), thus sparing patients from a toxic, costly, and potentially nonefficacious treatment. So far, widely used predictive markers of ICT response pertain to PD-L1 status by IHC, TMB, and **microsatellite status**, but these remain insufficient for many malignancies [77,78].

When taking the complexity of the immune system into consideration, it is becoming more evident that we will have to integrate through comprehensive multi-omic evaluations both genomic and nongenomic biomarkers in the predictive tools for ICT response/resistance, if all aspects of the cancer–immunity cycle [79] are to be encompassed in decision-making algorithms. Here, we have proposed 9p21 status as an emerging genomic marker candidate but further and robust mechanistic studies are required to dissect the associations of 9p21 loss and immune evasion (see Outstanding questions). These mechanistic approaches might also contribute to establishing accurate assays that can detect '9p21-ness' and pharmacological vulnerabilities of solid tumors, and which might contribute to enhancing the efficacy of future ICT in immunologically 'cold' tumors. The validation of the predictive value of this biomarker in the clinic would require its evaluation in additional pan-cancer as well as histology-specific data sets in patients treated with and without ICT. The current era of open data sharing has been conducive to generating hypothesis-driven questions. Studies such as INSPIRE, in which ICT outcomes are examined in genomic, transcriptomic, and histological granularity, can be instrumental in validating important scientific observations. To take it one step further, patients with 9p21 loss represent candidates for the investigation of novel immunotherapies or combination strategies that purport to activate host immunity despite a pre-existent 'cold' TME. Therefore, clinical trial designs incorporating this potential biomarker of resistance may be of therapeutic interest.

Outstanding questions

9p21.3 loss of heterozygosity does not lead to reduced CDKN2A/MTAP expression but is still associated with poor survival; to what extent do other key players modulated by the 9p21.3 locus drive this survival effect? More research into dissecting the role of 9p21 as a regulator of transcription is warranted.

Can pharmacological inhibition of cyclin-dependent kinase pathways in tumors with 9p21.3 loss enhance immune checkpoint inhibition? Clinical trials stratifying patients based on their 9p21.3 status will be required to address this question.

Can pharmacological blockade of the MTAP pathway via novel inhibitors of MTA and/or arginine methyltransferase augment immune checkpoint inhibition in tumors with 9p21.3 loss? Emerging research on the interplay between metabolome and epigenetic modulation will be imperative to answer this question.

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Declaration of interests

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Resource

[i]https://clinicaltrials.gov/ct2/show/NCT02644369

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