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Causal inference of the effect of plasma proteins on the incidence of oral cancer: two-sample Mendelian randomization

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Abstract

Objective This study is aimed to investigate the causal relationship between plasma proteins and oral cancer risk using two-sample MR (Mendelian randomization).

Methods Summary-level GWAS (genome-wide association study) data on plasma protein levels (4,907 proteins) and oral cancer (6,034 cases, 6,585 controls) of European ancestry were utilized. SNPs (single nucleotide polymorphisms) associated with proteins at genome-wide significance were selected as instrumental variables. Multiple MR methods including IVW (inverse-variance weighted), MR-Egger, weighted median, simple mode and weighted mode were applied to estimate causal effects. Sensitivity analyses were conducted.

Results Eight plasma proteins (CCDC167, MID2, NDRG4, PEAR1, PIAS4, RCAN1, SAMHD1 and TNMD) were identified to have significant causal associations with oral cancer risk. NDRG4, RCAN1, SAMHD1 and TNMD were associated with increased oral cancer risk while PEAR1 was associated with decreased risk. The causal estimates were consistent across different methods. Sensitivity analyses indicated the results were robust without significant heterogeneity or horizontal pleiotropy. Multivariable MR adjusting for smoking, alcohol intake and periodontal disease showed CCDC167, MID2, NDRG4, PEAR1, PIAS4 and SAMHD1 still had direct effects on oral cancer.

Conclusion This two-sample MR study provides evidence for potentially causal effects of several plasma proteins on oral cancer risk. The identified proteins may serve as biomarkers and shed light on biological mechanisms underlying oral carcinogenesis. Further research is warranted to validate and extend these findings.

Keywords Plasma proteins, Oral cancer, Two-sample Mendelian randomization, Causal inference

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Introduction

Oral cancer, comprising cancers of mucosa, alveolar ridge, tongue, hard palate, retromolar trigone, floor of the mouth and labial mucosa [1], was estimated to account for 377,713 new cases and 177,757 deaths worldwide in 2020 [2]. Identifying biomarkers can predict oral cancer risk and illuminating biological mechanisms is key for reducing the global burden of this disease [3]. Emerging evidence suggests dysregulated expression of plasma proteins may be implicated in oral carcinogenesis [4]. Plasma proteins play central roles in regulating signaling pathways involved in critical cancer processes including proliferation, apoptosis, angiogenesis and metastasis [5]. Dysregulation of plasma proteins can drive carcinogenesis by stimulating oncogenic cascades, disabling anti-tumor immune surveillance, increasing clotting and inflammation, modifying the TME(tumor microenvironment), disrupting normal structural barriers to invasion and metastasis [6]. Changes in plasma protein levels may occur before clinical diagnosis, making them promising biomarker candidates. The perturbed plasma protein homeostasis appears to enable key hallmarks of cancer causally. However, whether observed associations reflect causal and direct effects of plasma proteins on oral cancer development remains unclear.

MR (Mendelian randomization) has emerged as a powerful technique that utilizes genetic variants as instrumental variables to infer causality between exposures and outcomes [7]. By utilizing genetic variants as instrumental variables, MR can overcome limitations of conventional observational studies and strengthen causal inference. In cancer research, MR studies have provided evidence on the causal roles of various exposures in carcinogenesis including biomarkers like inflammatory proteins, lifestyle factors like smoking and alcohol, environmental exposures like air pollution [8]. MR studies on oral cancer have confirmed causal effects for known risk factors like smoking and alcohol consumption [9], obesity and related metabolic traits such as T2D (type 2 diabetes), hypertension and dyslipidaemia [10]. Recent large-scale MR studies leveraging GWAS (genome-wide association study) data have also identified causal associations between gene expression levels and cancer susceptibility [11]. MR's ability to probe causality utilizing observational data makes it an invaluable tool for elucidating biological mechanisms, identifying promising prevention and early detection targets to promote cancer research.

In this study, a two-sample MR study to investigate potential causal effects of plasma protein levels on oral cancer risk was performed. By integrating large-scale GWAS data on plasma protein levels and oral cancer susceptibility, it was aimed to provide robust causal evidence on the role of dysregulated plasma proteins in oral oncogenesis.

Materials and methods

Study reporting guidelines and study design

The aim of this study is to investigate the effect of plasma proteins on oral cancer using two-sample MR and public datasets. Study Reporting was performed according to the Strengthening the Reporting of Observational Studies in Epidemiology Using MR (The STROBE-MR Statement) [12]. Figure 1 shows a schematic diagram of the study design.

Data sources

GWAS data for plasma proteins: deCODE Genetics used Illumina SNPs (single nucleotide polymorphisms) chip to sequence the whole genome of 49,708 Icelanders and measured the plasma levels of 4907 proteins in 35,559 of them, finally carried out the GWAS analysis of proteins [13].

GWAS data for oral cancer: Corina Lesseur et al. conducted a GWAS of 6,034 oral and pharyngeal cancer cases and 6,585 controls from Europe, North America and South America, in which data on oral cancer were obtained from 4151 populations of European ancestry including 1223 oral cancer cases and 2928 controls [14].

Other GWAS data: GWAS data of ever smoked were from GWAS analysis data of UK Biobank, including 280,508 smokers and 180,558 controls [15]; The GWAS data of alcohol intake frequency were from the GWAS analysis data of alcohol intake frequency of 462,346 individuals in UK Biobank [15]; The GWAS data of periodontal disease (periodontitis) were derived from the GWAS analysis of 17,353 clinical periodontitis cases and 28,210 controls from a European population by Dmitry Shungin et al. [16].

Instrumental variable selection

A valid genetic variation instrumental variable must satisfy 3 core assumptions: (1) the associativity assumption, that is, the selected instrumental variable must be associated with the exposure factor significantly. (2) independence assumption, that is, the instrumental variable must not be significantly related to potential confounders that may affect the exposure or outcome. (3) exclusivity limitation, that is, the instrumental variable can only affect the outcome through the path of "instrumental variable → exposure → outcome".

In this study, the instrumental variable screening criteria for exposure were as follows: $P<5\times10^{-8}$ of SNPs in GWAS were used as the primary screening criteria; SNPs in linkage disequilibrium (SNPs with $r<0.01$ a^{2}nd physical distance > 10000 kb between each two genes) were excluded. Instrumental variables were extracted from

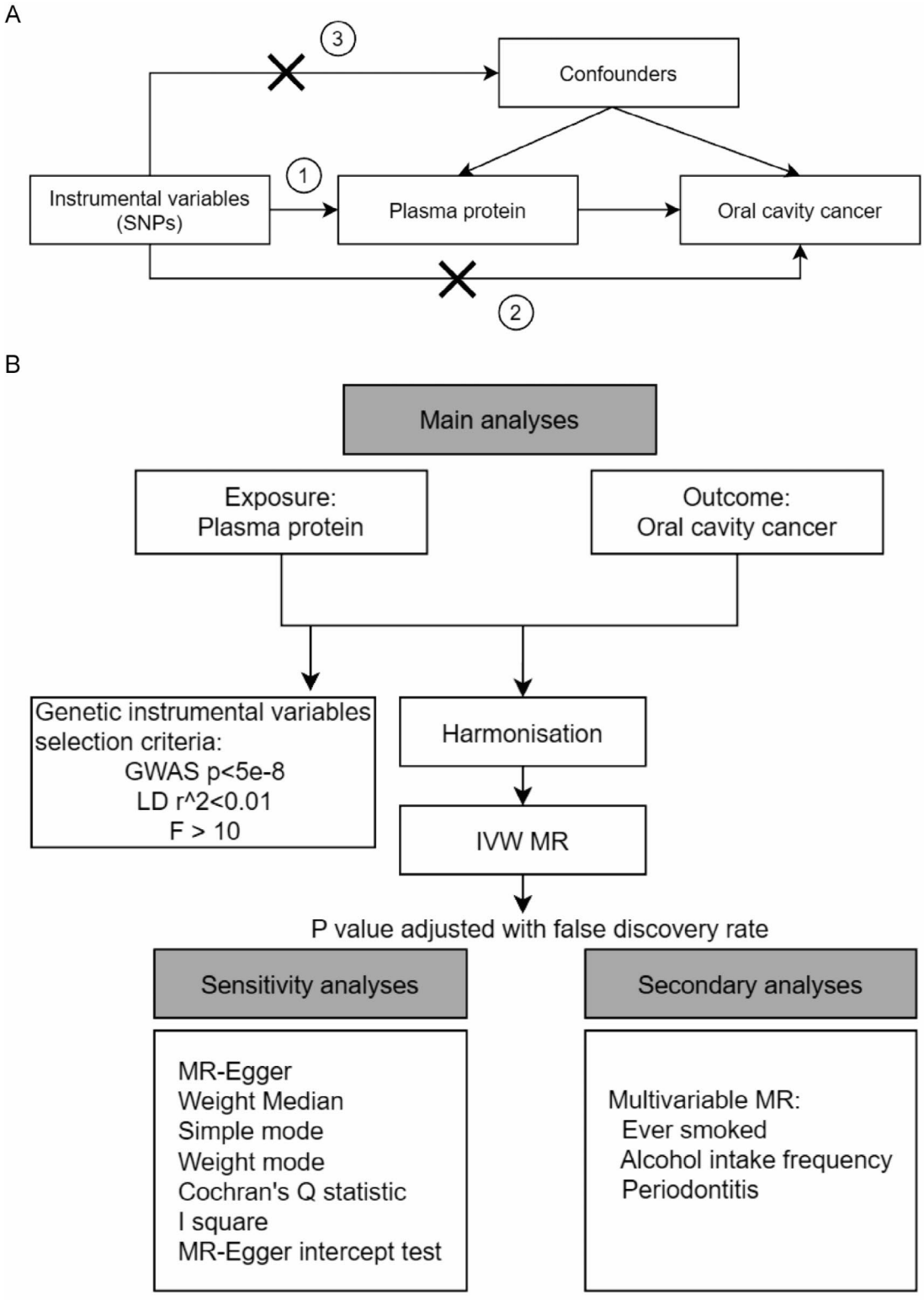

Fig. 1 The schematic diagram of the present research. (**A**) the basic assumptions of MR analysis, including (1) the association assumption, that is, the selected instrumental variable must be significantly related to the exposure factor; (2) independence assumption, that is, the instrumental variable must have no significant correlation with potential confounders that may affect the exposure or outcome; (3) exclusivity limitation, that is, the instrumental variable can only affect the outcome through the path of "instrumental variable → exposure → outcome". (**B**) Flow chart of the analytical methods of this study. SNP, single nucleotide polymorphism; IVW, inverse variance weighted; MR, Mendelian randomization; GWAS, genome-wide association study; LD, Linkage disequilibrium

GWAS of outcome data based on the selected SNPs. Also, F-statistics were calculated to assess weak instrumental variable bias. When F < 10, it indicates that the genetic variation used is a weak instrumental variable, which may have a certain bias on the results [17], so it should be removed to avoid affecting the results. The formula for calculating the F-statistic is as follows:

$$\mathrm{F} = \frac{N-k-1}{k} \times \frac{R^2}{1-R^2}$$

Where n is the sample size, k is the number of instrumental variables used and R^2 reflects the extent to which the instrumental variables explain the exposure. $R^2 = 2 \times (1\text{-MAF}) \times \mathrm{MAF} \times ^2\beta$, where MAF is the minimum allele frequency and β is the allele effect size.

MR Causal effect estimation

Various two-sample MR methods were used to assess causal effects of exposures on outcomes, including: IVW (Inverse-variance weighted), MR-Egger method, Weight Median method, simple mode and weight mode. Some studies have shown [18] that the IVW method was slightly stronger than others under certain conditions; Its characteristics are that the existence of intercept term is not considered in the regression and the inverse of the outcome variance is used as the weight for fitting. Therefore, in the absence of pleiotropy and with or without heterogeneity, the IVW method was used as the main MR Analysis, supplemented by the other 4 methods (IVW random effects model was used in the presence of heterogeneity). When pleiotropy is present, MR-Egger method is used to calculate the results.

Sensitivity analysis

The sensitivity analysis of the results was carried out by various methods such as heterogeneity test, pleiotropy test and one-by-one exclusion test, as follows:

(1) Heterogeneity test: The Cochran Q test was used to evaluate the heterogeneity among the SNP estimates, the Cochran Q test was statistically significant, indicating that the analysis results had significant heterogeneity. The random effect model of IVW was used to evaluate the causal effect size for highly heterogeneous results. The Cochran Q test could only test the presence or absence of heterogeneity, but could not test the distribution of heterogeneity. Therefore, the I^2 statistic was used to reflect the proportion of heterogeneous part of the instrumental variable in the total variation, and the result with high heterogeneity was estimated by the IVW random effect model: when $I^2 \leq 0$, it was set to 0, indicating that no heterogeneity was observed. If $I^2 = 0$–25%, it indicates mild heterogeneity. $I^2 = 25$-50%, indicating moderate heterogeneity; $I^2 > 50\%$ indicated high heterogeneity. The specific calculation formula is as follows:

$$\mathrm{I2} = \frac{Q-df}{Q} \times 100\%$$

(2) Pleiotropy test: MR-Egger method was used to test the pleiotropy of instrumental variables. If the *P* value of MR-Egger's intercept is less than 0.05, it indicates that there is significant horizontal pleiotropy of genetic variation.

(3) Leave-one-out test: The MR Results of the remaining instrumental variables were calculated by excluding single SNP one by one to assess whether the SNP affected the association between adiposity and cognitive function. If there is a large difference between the MR Effect estimates and the total effect estimates after excluding an instrumental variable, it indicates that the MR Effect estimates are sensitive to that SNP.

Statistical analysis

All data calculation and statistical analysis were performed using R programming (https://www.r-project.org/, version 4.3.0), and the TwoSampleMR package was mainly used for MR analysis [19]. Cochran Q test and leave-one-out analysis were used to evaluate the robustness and reliability of the results. MR-Egger intercept method was used to test genetic pleiotropy. The evaluation indicators were OR (odds ratio) and 95% CI (confidence interval). All statistical *P* values were two-sided. For SNPs generated from GWAS studies, $P < 5 \times 10^{-8}$ was considered statistically significant. For other statistical tests, $P < 0.05$ was considered statistically significant and FDR (false discovery rate) method was used to correct the *P* value for multiple tests.

As shown in Fig. 1, this two-sample MR study investigated the causal effect of plasma proteins on oral cancer risk using genetic instrumental variables. Genome-wide significant SNPs ($p < 5 \times 10^{-8}$) associated with plasma protein levels were selected from GWAS data as instrumental variables for the exposure (plasma proteins). These SNPs were harmonized with the outcome GWAS data (oral cancer) to extract corresponding effect estimates. The primary analysis was IVW MR to estimate causal effects of each plasma protein on oral cancer risk, with *p*-values adjusted by FDR. Sensitivity analyses included MR Egger, weight median, simple and weighted mode methods to assess consistency. Heterogeneity was evaluated by Cochran's Q statistic and I^2. Horizontal

Table 1 Selection of instrumental variables for plasma protein and oral cancer

Exposure	Number of SNPs	Median of F	Minimum of F	Maximum of F
CCDC167	4	58.57	37.1	83.63
MID2	3	32.92	31.98	93.94
NDRG4	6	42.24	30.09	125.42
PEAR1	22	45.51	30.67	758.74
PIAS4	3	44.04	35.75	50.28
RCAN1	5	59.62	50.09	104.33
SAMHD1	5	54.15	31.66	94.12
TNMD	4	51.45	31.8	85.54

Note SNPs, Single Nucleotide Polymorphisms; F, F statistics

pleiotropy was assessed by MR Egger intercept test. Leave-one-out analysis was performed to evaluate outlier SNPs. Secondary multivariate MR analyses adjusted for potential confounders including smoking, alcohol intake and periodontitis. Overall, this two-sample MR approach using genetic IVs allowed investigation of the causal effects of plasma protein biomarkers on oral cancer risk.

Results

Instrumental variable screening

According to the screening criteria of instrumental variables in this study, SNPs with linkage disequilibrium were removed. After matching with the GWAS data of oral cancer, SNPs related to plasma protein levels were included as instrumental variables. The number of instrumental variables of each protein is shown in Table 1. Due to the large number of exposures, only the indicators that were significant (P-FDR < 0.05) by MR Analysis are shown in Table 1. The F-test statistics of instrumental variables of these indicators were all greater than 10, indicating that most of the SNPs screened in this study were strong-effect instrumental variables, and the possible bias caused by weak instrumental variables was limited.

MR Causal effect estimation

5 models, MR Egger, Weighted median, Inverse variance weighted, Simple mode and Weighted mode were used for analysis and the results are shown in Fig. 2. IVW model results showed that CCDC167, MID2, NDRG4, PEAR1, PIAS4, RCAN1, SAMHD1 and TNMD all had significant causal associations with oral cancer, among which NDRG4 (OR = 3.32, p-FDR = 0.038), SAMHD1 (OR = 4.10, P = 0.038) and SAMHD1 (OR = 4.10, P = 0.038) were significantly associated with oral cancer. P-fdr = 0.021, RCAN1 (OR = 4.24, p-FDR = 0.021), CCDC167 (OR = 4.27, p-FDR = 0.045), TNMD (OR = 4.64, p-FDR = 0.021), MID2 (OR = 5.91, P < 0.05), RCAN1 (OR = 4.24, p-FDR = 0.021). P-FDR = 0.028, PIAS4 (OR = 6.96, P-FDR = 0.038) and PEAR1 (OR = 0.62, P-FDR = 0.021) had a lower risk of oral cancer. CCDC167 (Fig. 3A), MID2 (Fig. 3B), NDRG4 (Fig. 3C), PEAR1 (Fig. 3D), PIAS4 (Fig. 3E), RCAN1 (Fig. 3F), SAMHD1 (Fig. 3G) and TNMD (Fig. 3H). Most of the MR Analyses of the other models gave consistent direction estimates and the slopes were more consistent.

The forest plot shows the causal association analysis results of multiple MR models on plasma proteins and oral cancer. The estimated effect values are presented as OR and 95% CI. The number of instrumental variables used in each model, Beta values and standard errors are also shown.

Sensitivity analysis

Heterogeneity of the significant results was tested using the Cochran Q test and I^2 statistic, as shown in Table 2. The results showed that there was no heterogeneity in the MR Results of most significant plasma proteins for oral cancer (Cochran Q p-value > 0.05), there was moderate heterogeneity in the MR Results of RCAN1 for oral cancer (Cochran Q p-value = 0.019, I^2 = 45.96%). CCDC167 (Fig. 4A), MID2 (Fig. 4B), NDRG4 (Fig. 4C), PEAR1 (Fig. 4D), PIAS4 (Fig. 4E) and RCAN1 (Fig. 4F). The funnel plot of the instrumental variables of SAMHD1

Fig. 2 Analysis results of multiple models for the MR analysis of plasma proteins and oral cancer. MR, Mendelian randomization

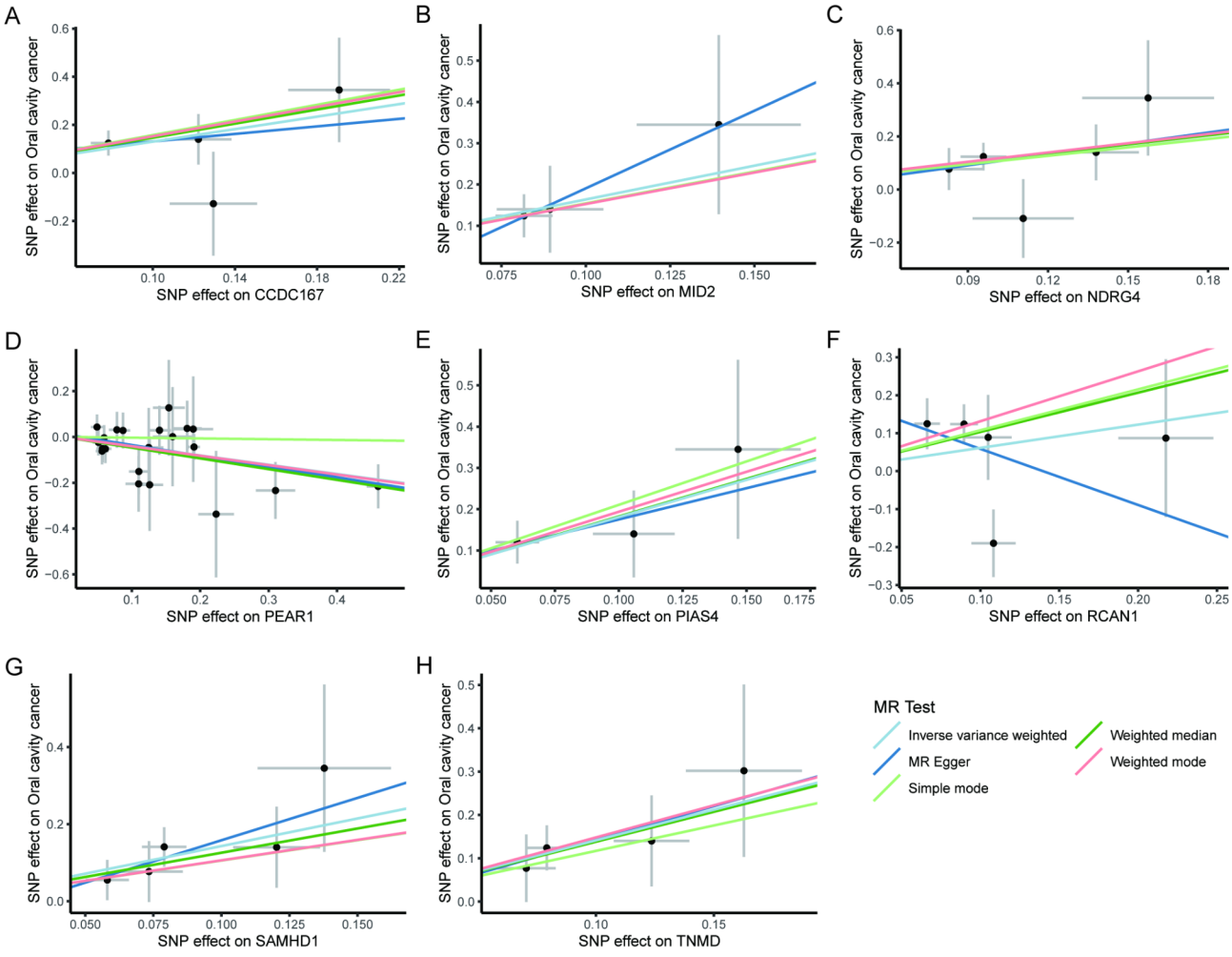

Fig. 3 Effect estimates of different models for MR analysis of plasma proteins and oral cancer. **A**–**G** Scatter plots show causality for CCDC167 (**A**), MID2 (**B**), NDRG4 (**C**), PEAR1 (**D**), PIAS4 (**E**), RCAN1 **(F)**, SAMHD1 (**G**), TNMD (**H**) and oral cancer, the slopes of the lines indicate the size of causality predicted by the different models. MR, Mendelian randomization

Table 2 MR analysis heterogeneity test for the association between plasma protein and oral cancer. MR, mendelian randomization

Exposure	Q	Q df	Cochran Q *p*-value	I^2 (%)
CCDC167	2.3	3	0.513	
MID2	0.33	2	0.849	
NDRG4	3.22	4	0.522	
PEAR1	11.77	20	0.924	
PIAS4	0.42	2	0.811	
RCAN1	11.75	4	0.019	45.96
SAMHD1	1.28	4	0.865	
TNMD	0.38	3	0.945	

Note Q, Cochran's Q test statistic; Q df, degrees of freedom for the Q test; I^2 statistic reflects the proportion of heterogeneity attributed to instrumental variables in the total variability

(Fig. 4G) and TNMD (Fig. 4H) showed that the scatter of causal association effects was basically symmetrical, indicating that there was no potential bias in the results.

MR-Egger regression was used to test the horizontal pleiotropy of instrumental variables. The statistical hypothesis test *P* values of the intercept terms of each index were greater than 0.05, the intercept was close to 0, indicating that the causal inference in this study was not affected by the horizontal pleiotropy (see in Table 3).

Sensitivity analysis was performed using the one-by-one exclusion test. CCDC167 (Fig. 5A), MID2 (Fig. 5B), NDRG4 (Fig. 5C), PEAR1 (Fig. 5D), PIAS4 (Fig. 5E), RCAN1 (Fig. 5F) and SAMHD1(Fig. 5G) were not found, TNMD (Fig. 5H) effect estimates changed in significance, suggesting the stability of the results.

Multivariate MR analysis

The direct effects of significant plasma proteins on the risk of oral cancer were evaluated by multivariable MR

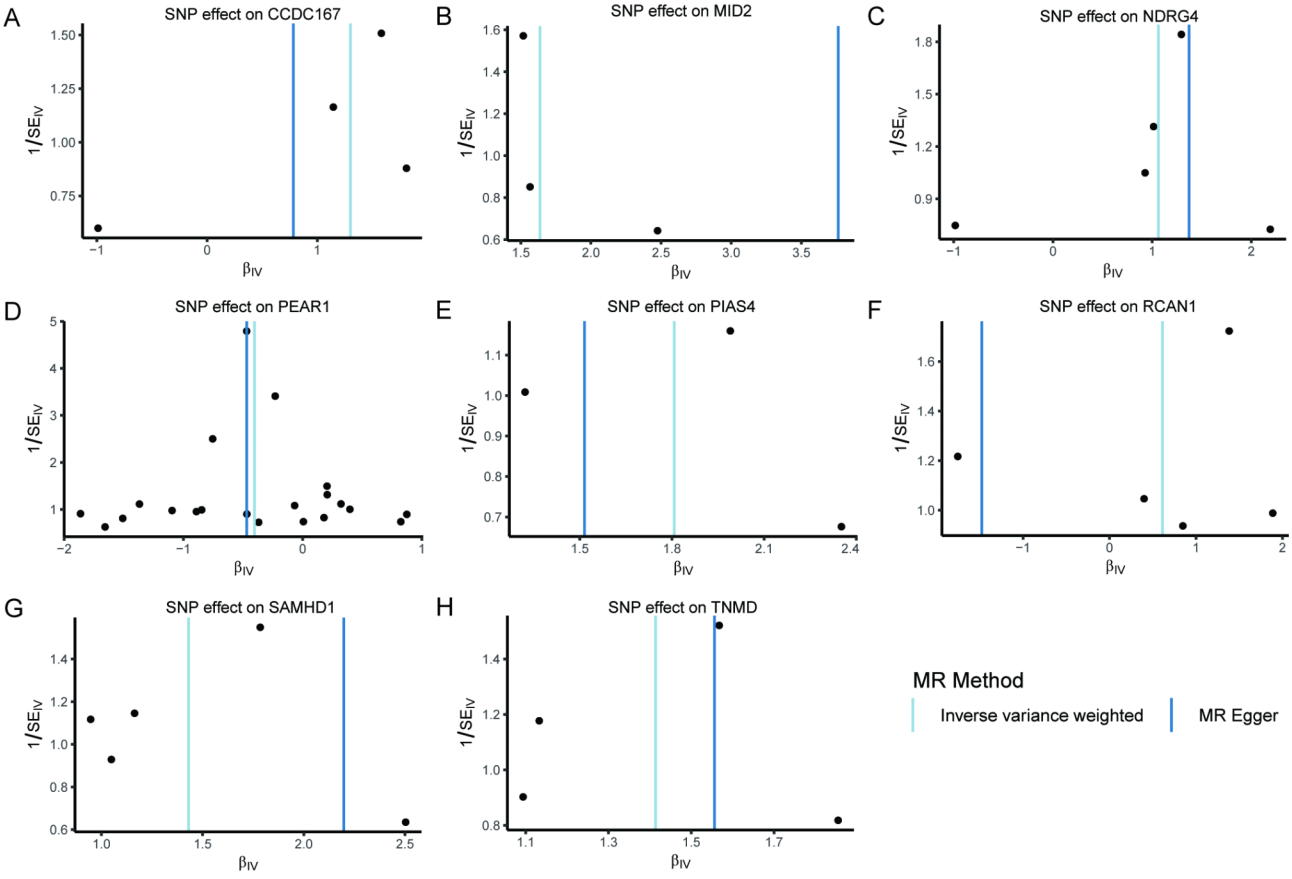

Fig. 4 Funnel plot of heterogeneity test for MR analysis of plasma proteins and oral cancer. **A**–**G** Funnel plot showing causal effect estimates for each instrumental variable of CCDC167 (**A**), MID2 (**B**), NDRG4 (**C**), PEAR1 (**D**), PIAS4 (**E**), RCAN1 (**F**), SAMHD1 (**G**), TNMD (**H**) and oral cancer, Causal effect estimates from Inverse variance weighted and MR Egger models are labeled with lines on the plots. MR, Mendelian randomization

Table 3 MR analysis of horizontal pleiotropy for the association between plasma protein and oral cancer. MR, mendelian randomization

Exposure	MR-Egger intercept	Standard error	*P* value
CCDC167	0.052	0.163	0.779
MID2	0.186	0.331	0.674
NDRG4	0.032	0.209	0.887
PEAR1	0.012	0.028	0.680
PIAS4	0.024	0.146	0.898
RCAN1	0.206	0.221	0.418
SAMHD1	0.062	0.126	0.658
TNMD	0.013	0.154	0.94

Note MR, Mendelian randomization

Analysis with exposure to smoking, drinking frequency and periodontal disease separately. After adjusting for the indirect effect of smoking, model 1 showed that CCDC167, MID2, PEAR1, SAMHD1 and TNMD still had significant effects on oral cancer ($P<0.05$). After adjusting for the effect of drinking frequency, model 2 showed that CCDC167, MID2, NDRG4, PEAR1, PIAS4, SAMHD1 and TNMD had significant direct effects ($P<0.05$). Model 3 showed significant direct effects of CCDC167, MID2, PEAR1, PIAS4, SAMHD1 and TNMD after adjusting for the effect of periodontal disease ($P<0.05$). After adjusting for the indirect effects of smoking, drinking frequency and periodontal disease, model 4 showed that CCDC167, MID2, NDRG4, PEAR1, PIAS4 and SAMHD1 still had direct effects on oral cancer ($P<0.05$) (Table 4).

Discussion

There are some research findings regarding the relationship between plasma proteins and oral cancer through searching the literature database. Some studies support a causal relationship between CRP (C-Reactive Protein) levels and increased risk of oral and oropharyngeal cancers [20]. Elevated plasma levels of CLIC1 (Recombinant Chloride Intracellular Channel Protein 1) have been demonstrated in only 2 cancer types, namely nasopharyngeal carcinoma and OSCC (Oral Squamous Cell Carcinoma) [21], CLIC1 plasma concentrations were significantly higher in metastatic OSCC patients than in non-metastatic patients ($p<0.0001$) [22]. Significant differences in plasma amounts of apolipoprotein A-IV and the ratio of LRG1 (Leucine-rich alpha-2-glycoprotein 1)

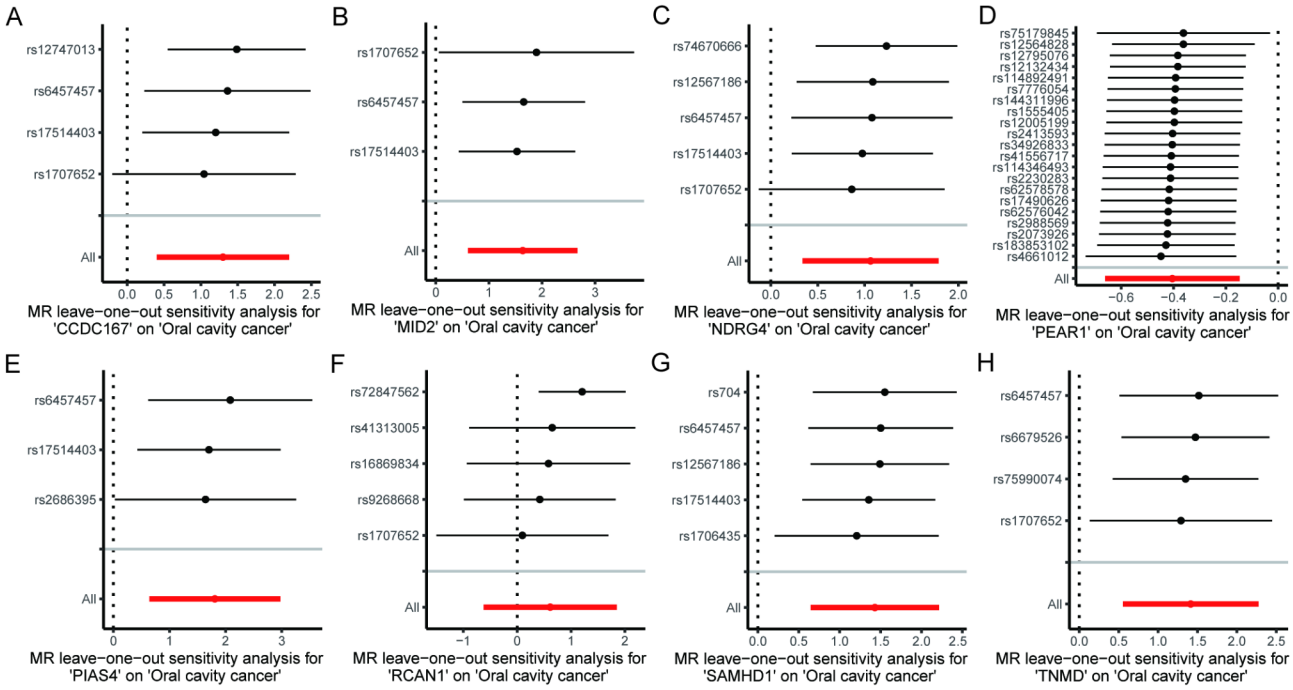

Fig. 5 MR leave-one-out analysis of plasma proteins and oral cancer. **A**–**G** Funnel plot showing causal effect estimates of CCDC167 (**A**), MID2 (**B**), NDRG4 (**C**), PEAR1 (**D**), PIAS4 (**E**), RCAN1 (**F**), SAMHD1 (**G**), TNMD (**H**) and oral cancer after SNP exclusion one by one. The causal effect estimates of the Inverse variance weighted model are labeled in red on the plot. MR, Mendelian randomization

to total protein between normal plasma and oral cancer plasma [23]. Plasma IL6 (Interleukin-6) levels are significantly elevated in OSCC patients and correlate with tumor stage [24, 25]. Interference with NDRG1 and its upstream proteins may rescue NDRG function as a potential therapeutic strategy to prevent oral cancer progression to metastasis [26]. However, it has also been suggested that saliva may be more suitable than blood for protein biomarker-based oral cancer detection [27]. It was confirmed that 8 plasma proteins-CCDC167, MID2, NDRG4, PEAR1, PIAS4, RCAN1, SAMHD1 and TNMD had significant effects on OSCC in the study.

A series of sensitivity analyses have been performed in this study. Heterogeneity test: the Cochran Q test and I^2 statistic revealed that most of the SNPs exhibited low heterogeneity among themselves, suggesting a consistent effect of different genetic variants on oral cancer risk. Multiplicity test (MR-Egger method): the MR-Egger regression revealed that the intercept term was close to 0 with $p>0.05$, implying that the study results were not affected by unmeasured multiplicity bias. Thus, the causal interpretation of the effect of plasma protein levels on oral cancer is strengthened. Leave-one-out: By removing each SNP one by one and reanalyzing the results, little change was found in the results after removing any single SNP, demonstrating the insensitivity of the MR analysis results to the effect of a single genetic variant, thus increasing confidence in the results.

This two-sample MR study identified several plasma proteins to have potentially causal effects on oral cancer risk. Specifically, NDRG4, RCAN1, SAMHD1 and TNMD were found to be associated with increased risk while PEAR1 was associated with decreased risk of oral cancer. These results were robust across different statistical methods and sensitivity analyses. Multivariable analyses adjusting for smoking, alcohol intake and periodontal disease also showed that CCDC167, MID2, NDRG4, PEAR1, PIAS4 and SAMHD1 had direct effects on oral cancer independent of these factors. Overall, this study provides novel evidence that alterations in levels of specific plasma proteins may influence oral carcinogenesis. The identified proteins may serve as useful biomarkers and shed light on biological mechanisms linking plasma protein dysregulation to development of oral cancer.

CCDC proteins have diverse regulatory functions in platelets, for example, the regulatory role of CCDC26 in platelet aggregation and activation; the promoting role of CCDC88A in platelet integrin αIIbβ3 activation; the involvement of CCDC6 in megakaryopoiesis and proplatelet formation; the regulatory role of CCDC152 in platelet dense granule secretion and PF4 (platelet factor 4) release. There is still no report showing the mediating role of CCDC167 gene in the function of platelet. CCDC167 has been demonstrated to be a driver of breast tumorigenesis and progression through its impacts on

Table 4 The results of multivariable MR analysis on the impact of plasma protein and oral cancer. MR, mendelian randomization

Model	Exposure	Number of SNPs	OR (95%CI)	*P* value
Model 1	CCDC167	4	2.45 (1.03, 5.83)	0.044
	MID2	3	3.21 (1.22, 8.46)	0.018
	NDRG4	6	1.73 (0.83, 3.59)	0.144
	PEAR1	19	0.66 (0.50, 0.87)	0.004
	PIAS4	3	2.53 (0.87, 7.32)	0.087
	RCAN1	4	1.51 (0.68, 3.35)	0.312
	SAMHD1	5	2.57 (1.17, 5.65)	0.018
	TNMD	4	2.36 (1.01, 5.56)	0.049
Model 2	CCDC167	4	2.63 (1.25, 5.53)	0.011
	MID2	2	3.62 (1.42, 9.18)	0.007
	NDRG4	5	2.41 (1.27, 4.58)	0.007
	PEAR1	20	0.66 (0.51, 0.85)	0.001
	PIAS4	3	3.93 (1.68, 9.17)	0.002
	RCAN1	4	1.71 (0.80, 3.65)	0.165
	SAMHD1	5	3.22 (1.64, 6.32)	0.001
	TNMD	4	2.50 (1.21, 5.15)	0.013
Model 3	CCDC167	4	4.27 (1.64, 11.14)	0.003
	MID2	3	5.55 (3.42, 9.02)	< 0.001
	NDRG4	6	2.19 (0.67, 7.13)	0.193
	PEAR1	21	0.67 (0.55, 0.82)	< 0.001
	PIAS4	3	6.56 (6.54, 6.58)	< 0.001
	RCAN1	4	1.67 (0.25, 11.25)	0.598
	SAMHD1	5	4.18 (2.47, 7.07)	< 0.001
	TNMD	4	4.19 (2.99, 5.88)	< 0.001
Model 4	CCDC167	4	2.06 (1.02, 4.17)	0.044
	MID2	2	2.50 (1.06, 5.89)	0.037
	NDRG4	5	2.01 (1.07, 3.74)	0.029
	PEAR1	19	0.66 (0.50, 0.86)	0.002
	PIAS4	3	2.47 (1.12, 5.49)	0.026
	RCAN1	4	1.58 (0.78, 3.18)	0.204
	SAMHD1	5	2.48 (1.29, 4.76)	0.006
	TNMD	4	1.83 (0.91, 3.65)	0.088

Note Model 1, Multivariable MR analysis of plasma protein and ever smoked on oral cavity cancer; Model 2, Multivariable MR analysis of plasma protein and alcohol intake frequency on oral cavity cancer; Model 3, Multivariable MR analysis of plasma protein and periodontitis on oral cavity cancer; Model 4, Multivariable MR analysis of plasma protein, ever smoked, alcohol intake frequency, and periodontitis on oral cavity cancer. MR, Mendelian randomization; SNPs: Single Nucleotide Polymorphisms

sustaining proliferation, resisting apoptosis and promoting invasion of MCF-7 breast cancer cells [28]. Pathway analysis of CCDC167-coexpressed genes in breast cancer revealed enrichment in cell cycle control and ubiquitination networks [28]. Clinically, elevated CCDC167 predicted poorer prognosis and survival outcomes in breast cancer patients [28], however, the expression pattern and potential regulatory role of CCDC167 in oral cancer is unknown.

The MID2 gene encodes a ubiquitin ligase protein involved in the ubiquitination and degradation of proteins. The specific mechanisms by which MID2 regulates platelet production are still being investigated, it likely has to do with its role in ubiquitination which is important for megakaryocyte development and platelet formation. MID2 has also been implicated as a potential oncogene, as its overexpression has been observed in several cancer types [29]. Proposed cancer-promoting mechanisms of elevated MID2 include the activation of mTOR and Wnt/β-catenin signaling, which is known to be involved in oncogenesis [30]. MID2 is thought to be involved in processes like epithelial-mesenchymal transition, cell cycle regulation and apoptosis in HCC (hepatocellular carcinoma) along with the MID1/α4/PP2A protein complex [29]. However, no studies have yet elucidated the specific regulatory mechanisms or pathways by which MID2 contributes to oral cancer phenotypes.

PEAR1 (Platelet Endothelial Aggregation Receptor 1) is a membrane protein expressed on platelets and endothelial cells, plays a role in platelet aggregation and endothelial cell activation [31]. PEAR1 activates the platelet integrin αIIbβ3, leading to increased platelet aggregation [32]. Additionally, PEAR1 expressed on endothelial cells positively regulates PTEN, which is a tumor suppressor gene inhibiting tumor cells proliferation [33]. Silencing of PEAR1 was found to reduce PTEN and increase Akt signaling, thereby promoting angiogenesis and further supporting tumor growth, metastasis [33]. PEAR1 was found to be downregulated in several cancers including colorectal cancer [33], breast cancer [34] and acute myeloid leukemia [35]; however, its investigation in oral cancer is lacking.

PIAS4 (Protein Inhibitor Of Activated STAT 4) stands for activated protein inhibitors. The PIAS4 protein levels were significantly increased in pancreatic cancer and hepatocellular carcinoma [36, 37]. PIAS4 acts as an oncogene in pancreatic cancer by interacting with the tumor suppressor VHL, leading to VHL inactivation and upregulating HIF1α transcriptional activity [36]. PIAS4 was also found to promote tumorigenicity and metastasis of HCC cells by promoting the protein posttranslational modification SUMOylation of AMPKαand NEMO [37]. However, there is currently no evidence elucidating the regulatory role of PIAS4 specifically in the context of oral cancer.

SAMHD1 (sterile alpha motif and HD domain-containing protein-1) is a dNTP triphosphohydrolase enzyme that regulates the intracellular dNTP pool by catalyzing the hydrolysis of dNTPs to deoxynucleosides and tripolyphosphate [39]. The dNTP hydrolysis activity of SAMHD1 was found to regulate platelet activation and reactivity [39]. SAMHD1 is frequently downregulated in various cancer types, such as chronic lymphocytic leukemia, lung cancer, and colorectal cancer [40–42]. Downregulation or inactivation of SAMHD1 can result in elevated dNTP levels, and lead to increased DNA synthesis and replication stress; thereby contributing to genetic instability, DNA damage accumulation, and an

increased risk of cancer development. SAMHD1 acts as a tumor suppressor by inhibiting cell proliferation, promoting apoptosis, and preventing DNA damage and genomic instability [40–43]. SAMHD1 modulates the chemosensitivity to anticancer drugs by affecting the nucleotide metabolism and the innate immune response [44]. SAMHD1 interacts with several signaling pathways involved in cancers, such as EGFR/MAPK, Notch, PI3K/AKT, TGF-β, and Wnt pathways, and influences their activity and function [45]. Although many research have observed the altered expression of SAMHD1 in multiple malignancies, the investigation regarding SAMHD1's regulatory role in oral cancer is still lacking.

TNMD (Tenomodulin) is a type II transmembrane glycoprotein highly expressed in tendons/ligaments, also involved in extracellular matrix remodeling and cell adhesion [46]. TNMD expression was found to be significantly lower in OSCC tumors than in normal oral tissue, suggesting that TNMD may have a tumor suppressive function [47]. TNMD expression was negatively correlated with RFS (recurrence-free survival) in patients with OSCC, indicating that TNMD may be a prognostic marker or a potential therapeutic target for oral cancer [47]. The research regarding TNMD in cancers is rare and warrants to be further investigated.

There may be overlapping samples when using different GWAS datasets, which may lead to bias in MR estimates. Due to the aggregated nature of summarizing statistics, performing individual-level overlap checks was not feasible in this study. Moderate heterogeneity in the association of RCAN1 with oral cancer (Cochran Q=11.75, p=0.019, I^2 = 45.96%) was observed, which may imply that the effects of genetic instrumental variants on outcomes are not entirely homogeneous and may be caused by pleiotropic effects or differences in population structure. Therefore, random-effects IVW model and MR-Egger regression test were used to adjust for possible effects, which showed a small effect of pleiotropy (intercept close to 0, p=0.418). Future studies should maximize the use of individual-level data to ensure that samples do not overlap, thus providing more robust causal estimates. In addition, leave-one-out sensitivity analysis was used to evaluate the impact of potential outliers on MR results. The analysis showed that no single SNP had a disproportionate effect on the overall estimates of the association of CCDC167, MID2, NDRG4, PEAR1, PIAS4, RCAN1, SAMHD1 and TNMD with oral cancer, the funnel plot showed a symmetrical distribution of the effects of each SNP, which suggests that the results were not affected by systematic bias or outliers.

The majority of these proteins identified in the present research were found to regulate key aspects of platelet biology, for example, production, activation, secretion and aggregation. The aggregated platelets release growth factors like PDGF, VEGF and TGF-beta that stimulate angiogenesis to supply the tumor with nutrients and oxygen [48]. Platelets also coat cancer cells to shield them from immune attack by natural killer cells. Furthermore, platelets facilitate extravasation of cancer cells out of blood vessels by increasing vascular permeability, allowing invasion into surrounding tissues. The platelet-rich thrombi provide a scaffold for adhesion and growth of circulating tumor cells at distant sites. In the TME, platelets secrete inflammatory cytokines that enhance chronic inflammation and oxidative stress which fuels cancer promotion. Additionally, platelets are a source of TGF-beta which can switch immune cells to an immunosuppressive phenotype, further evading anti-tumor immunity. Through these myriad mechanisms, the altered expression of these platelet proteins in cancer cells activates platelets to establish a pro-angiogenic and immunosuppressive TME optimized for growth, metastasis and survival [49]. Therefore, the platelet proteins identified in the present research can be regarded as emerging therapeutic targets, as targeting their activity may disrupt the procarcinogenic activation of platelets.

The identification of several plasma proteins with potentially causal effects on oral cancer risk has important implications for future research. The results provide a rationale for pursuing functional studies to validate the mechanistic roles of these proteins in oral carcinogenesis and determine how they interact with known oncogenic pathways. Cell line and animal model experiments focused on the specific candidates like CCDC167, MID2 and SAMHD1 could elucidate their contributions to tumor development and growth at the molecular level. These proteins also represent promising biomarker targets - large-scale longitudinal studies could evaluate their utility for early detection and prognosis of oral cancer. Exploring interventional strategies modulating levels of these proteins may uncover novel therapeutic approaches. Finally, expanding this line of causality research to other cancer types and integrating diverse omics data could provide a more holistic perspective on the involvement of the plasma proteome in oncogenesis. Overall, these findings open several new research avenues to further understand the causal role of plasma protein dysregulation in oral cancer.

While this two-sample MR study provides evidence for potentially causal roles of plasma proteins in oral cancer, there are some limitations to consider. As a MR study, it relies on assumptions that the genetic variants used as instrumental variables are reliably associated with the exposure, not confounded, and only related to the outcome through the exposure. Violations of these assumptions can bias causal estimates. In this study, the influence of genetic factors on the risk of oral cancer was aimed to be investigated. The importance of possible

confounders was fully recognized such as dietary habits, socioeconomic status and coexisting diseases when analyzing disease risk. However, limited by the lack of information on these specific factors in the public GWAS dataset, it was unable to include them in this study. When multivariate MR analyses were performed, a balance must be found between the number of confounders included and the complexity of the statistical model. Theoretically, although including more confounders can improve the accuracy of the analysis, it may also make the model too complex and further cause overfitting problems due to the limitation of genetic instrumental variables as well as sample size constraints, especially when the number of genetic instrumental variables is limited. Therefore, in the current study, to ensure the precision of the analysis, a few major confounders that are expected to be associated with oral cancer risk based on existing knowledge were chosed and are more adequately supported by data. The statistical power of MR analyses was based on sample size, effect sizes and explained variance (expressed as F-statistics). The F-statistics for all instrumental variances were significantly greater than 10, avoiding the risk of weak instrumental variance bias. The statistical power of MR analyses is shown in Appendix 1. There are several SNPs with low power, which may be due to limitations such as sample size. However, there are several SNPs with relatively high power compared to others (e.g., rs75179845 and rs4661012), suggesting a potentially important role for these SNPs in oral cancer. The inclusion of data from diverse populations is essential for understanding the causal effects of plasma proteins on oral cancer risk across ethnicities. Unfortunately, limited by the diversity of data in currently available public GWAS databases, which lack sufficient data from populations of non-European origin, it was not able to achieve an analysis of a wider range of ethnic groups in this study. Population stratification could influence results, though this was addressed by focusing on European ancestry groups. Residual pleiotropy undetected by sensitivity analyses may affect findings. Finally, the study design determines associations at the population level-further cell and animal model functional studies are needed to definitively confirm causality for the specific protein candidates identified. Despite limitations, this study offers valuable insight into causal roles of plasma proteins in oral oncogenesis.

Conclusion

In summary, this two-sample MR study provides novel evidence that altered plasma levels of specific proteins may play causal roles in the development of oral cancer. By integrating large-scale GWAS data on plasma proteins and oral cancer, applying multiple MR methods, CCDC167, MID2, NDRG4, PEAR1, PIAS4, RCAN1, SAMHD1 and TNMD were identified to have significant causal associations with oral cancer risk. These results were robust in sensitivity analyses. The findings shed light on the involvement of plasma protein dysregulation in oral carcinogenesis and provide leads on potential functional mechanisms, biomarkers, and therapeutic targets. Overall, this study demonstrates the utility of MR for elucidating causal roles of plasma proteins in cancer etiology and highlights candidates for further investigation to elucidate their contributions to tumor development and growth at the molecular level.

Abbreviations

TME	Tumor microenvironment
MR	Mendelian randomization
T2D	Type 2 diabetes
GWAS	Genome-wide association study
SNPs	Single nucleotide polymorphisms
IVW	Inverse-variance weighted
OR	odds ratio
CI	Confidence interval
FDR	False discovery rate
CRP	C-Reactive Protein
CLIC1	Recombinant Chloride Intracellular Channel Protein 1
OSCC	Oral Squamous Cell Carcinoma
LRG1	Leucine-rich alpha-2-glycoprotein 1
IL6	Interleukin-6;PF4, platelet factor 4
HCC	Hepatocellular carcinoma
PEAR1	Platelet Endothelial Aggregation Receptor 1
PIAS4	Protein Inhibitor Of Activated STAT 4
SAMHD1	sterile alpha motif and HD domain-containing protein-1
TNMD	Tenomodulin; RFS, recurrence-free survival
LD	Linkage disequilibrium

Supplementary Information

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Supplementary Material 1

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Author contributions

TT conceptualized the research, conducted the analysis, interpreted the results and wrote the manuscript. TT, SL, S Hu and YZ were involved in the data analysis and results interpretation. GS, AA and S Huang administrated and supervised the whole research project. All authors read and approved the final manuscript.

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Data availability

The data presented in the study is provided within the manuscript, further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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