Research on the application of molecular epidemiology in the prediction and prognosis of cancer

PhD Thesis

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Abstract

Background: Molecular epidemiology is a subject that integrates molecular biology and basic epidemiology. It introduces biomarkers that are involved in the molecular pathway and specific genes related to disease risk into epidemiology to recognize disease causation, risk factors, prevention, and even treatment. It emphasizes the interactions between genetic, environmental and other factors that result in diseases. Cancer is a group of diseases caused by abnormal cells creating rapidly and uncontrollably and spreading to other organs. It is the world's second leading cause of death. Cancer is caused by a combination of genetic and environmental factors.

Molecular epidemiology provides tools for understanding the interaction between these factors. Different from the "traditional epidemiology" that revolves around time, place, and person, molecular epidemiology pays attention to looking for biomarkers related to diseases, such as DNA, transcription factors, RNA, cell surface receptors, enzymes, and even metabolites, and then uses them to explain the diseases' mechanisms in populations. According to the stages from exposure to cancer development, biomarkers used in cancer molecular epidemiology research can be categorized into markers of exposure, internal does markers, biologically effective does markers, early biological effect markers, altered structure/function markers, biomarkers of prognosis, and markers of disease. Those markers play an important and essential role in the diseases' early detection, diagnosis, staging, treatment, and prognosis.

However, as a novel field, how molecular epidemiology is used in specific practical studies, and how it helps to discover causes, explore risk factors, and ultimately protect, still remains an unclear explanation. Hence, the aim of this study is to display how molecular epidemiological runs in cancer research through two practical studies: ‘The relationship between single nucleotide polymorphisms and skin cancer susceptibility’ and ‘The treatment effect of hydrogen gas on lung cancer’.

Lung cancer (LC), as one of the most common cancers, causes increased numbers of

morbidity and mortality all over the world. The long intergenic non-protein coding RNA TP53-induced transcript (LINC-PINT) and lincRNA-P21 are TP53-induced transcripts. It was investigated that the expression of LINC-PINT and lincRNA-P21 decreased in the malignant cancer. Molecular hydrogen (H_2) is a new medical gas that is used as a selective antioxidant in the anti-inflammation and anti-apoptosis functions modulating. H_2 also plays a role in the LC treatment. Therefore, the first example aims to explore the effect of H_2 in lung cancer cells by identifying the expression of LINC-PINT and lincRNA-P21.

Risk assessment is the earliest evidence of impending cancer in persons who don't have cancer. Single nucleotide polymorphisms (SNPs) interfere with the function of certain genes and thus may influence the probability of skin cancer (SC). The correlation between SNPs and skin cancer lacks statistical power, however. Therefore, the purpose of the second example is to identify the gene polymorphisms involved in skin cancer susceptibility using network meta-analysis, and to determine the relationship between SNPs and SC risk.

Method: In the treatment biomarkers example, we employed qRT-PCR to assess the expression of LINC-PINT and lincRNA-P21 in lung cancer cells after 0%, 5% and 10% hydrogen gas treatment in the three time-groups.

In the early detection biomarkers example, we selected 59 studies investigating 275 SNPs associated with skin cancer using the following genotype and phenotype models: the alleles model (A vs. B), the dominant model (AA+AB vs. BB), and the recessive model (AA vs. AB+BB). We constructed further networks for selected SNPs by analyzing the results of the direct and indirect associations for each comparison of SNPs. In addition, p scores obtained from the network were utilized to select the SNPs with the highest chances of association with skin cancer.

Results: In the treatment biomarkers example, the main results we investigated are: 1) The expression of LINC-PINT and lincRNA-P21 upregulation with the treatment

concentration of H_2 gas; 2) LINC-PINT expression decreased in a relatively long H_2 treatment time; 3) The expression of lincRNA-P21 declined with the H_2 treatment.

In the early detection biomarkers example, the main results we certified are: 1)The minor alleles of rs2228570 (FokI) and rs13181(ERCC2) were associated with skin cancer and 2) Wildtype and heterozygous genotypes of rs475007 (MMP1) and the mutated homozygous genotype of rs238406 (ERCC2) were most likely to be associated with skin cancer.

Conclusions: These two real research examples explained how molecular epidemiology works. Hence, we can conclude that the SNP rs2228570 (FokI), rs13181(ERCC2), rs475007 (MMP1) and rs238406 (ERCC2) can be employed as the early biomarkers for skin cancer. And LINC-PINT and lincRNA-P21 have a possibility that being used as potential treatment biomarkers for lung cancer.

1 Content

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List of abbreviations

AD	atopic dermatitis
ANOVA	One-way analysis of variance
ATM	ataxia–telangiectasia mutated kinase
ATP	adenosine triphosphate
ATR	ataxia-telangiectasia
AUROC	area under the summary receiver operating characteristic curve
BCC	basal cell carcinoma
CA	chromosomal aberration
CI	confidence intervals
CM	cutaneous melanoma
cOR	combined OR
CS	Cockayne's syndrome
CSU	chronic spontaneous urticaria
ctDNA	Circulating tumour DNA
DMEM	Dulbecco's MEM
DOR	diagnostic OR
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GCDFP-15	gross cystic disease fluid protein-15
GWAS	Genome-Wide Association Studies
H_2	hydrogen
HB	hospital-based
hnRNP K	heterogeneous nuclear ribonucleoprotein K
Ho mut	mutated homozygous
HO-1	heme oxygenase-1
Ht	heterozygous
IDT	Integrated DNA Technologies
KASP PCR	Kompetitive allele specific PCR

LC	lung cancer
LINC-PINT	long intergenic non-protein coding RNA p53-induced transcript
lncRNA	long non-coding RNAs
LR	likelihood ratio
LRTChi2	Likelihood ratio test in joint model
LSD	Fisher's Least Significant Difference
MC1R	melanocortin receptor 1
MDM2	mouse double minute 2 homolog
MMPs	matrix metalloproteinases
MQ	methylene quinuclidinone
MSH	melanocyte-stimulating hormone
NMA	network meta-analysis
NMSC	nonmelanoma skin cancer
NOS	Newcastle-Ottawa Scale
Nrf2	nuclear factor erythroid 2-related factor 2
NSCLC	non-small-cell lung carcinoma
ORs	odds ratio
PAHs	polycyclic aromatic hydrocarbons
PB	population-based
PCR-RFLP	PCR-based restriction fragment length polymorphism
PDCD4	programmed cell death 4
PSA	prostate-specific antigen
PSRF	Potential Scale Reduction Factor
PUMA	TP53 upregulated modulator of apoptosis
RT-PCR	Real-Time PCR
SC	skin cancer
SCC	squamous cell carcinoma
SCLC	small-cell lung carcinoma

SMD	standardized mean differences
SNPs	Single nucleotide polymorphisms
SOD	superoxide dismutase
SSP-PCR	PCR with sequence-specific primers
SUCRA	surface under the cumulative ranking curve
TTD	trichothiodystrophy
UN	unknown
UVB	ultraviolet B
VDR	vitamin D receptor
Wt	wildtype
XP	xeroderma pigmentosum

1 Introduction

1.1 What is molecular epidemiology

"Epidemiology" was first defined to describe the study of epidemics by a Spanish physician in 1802 (1). However, the history of epidemiology can be traced back to the 16th century and was famous for John Snow's investigations into the causes of cholera epidemics in the 19th century (2). Early epidemiology was mainly applied to infectious diseases, but modern epidemiology includes not only diseases but also all health-related states. Epidemiology is therefore defined as: “The study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to control of health problems” (John M Last. A dictionary of Epidemiology (4th Ed) Oxford University Press (New York) 2001, p62) (3).

Although epidemiology has been known, molecular epidemiology, one of its subdivision, was not introduced until 1973 by Kilbeurne (4). And then molecular epidemiology was introduced in more detail in biomarkers, measurement and mechanisms until 1993 by Schulte and Perera (5). With the advancement of molecular biology techniques, such as the enzyme-linked immunosorbent assay (ELISA), electrochemistry, microfluidics and surface plasmon resonance sensors, protein, colourimetric, electrochemical assay, and microarray and so on (6), molecular epidemiology has gradually noted and applied.

"Molecular epidemiology" hasn't a uniform definition. However, it has a consensus understanding, that: Molecular epidemiology is a subject that integrates molecular biology and basic epidemiology. It introduces biomarkers that are involved in the molecular pathway and specific genes related to disease risk into epidemiology to recognize disease causation, risk factors, prevention, and even treatment. It emphasizes the interactions between genetic, environmental and other factors that result in disease (5,7,8).

1.2 What is cancer

Cancer is a group of diseases caused by abnormal cells proliferating expression rapidly and uncontrollably and spreading to other organs (9). Cancer is induced by a combination of genetic and environmental factors. It is the world's second most important cause of death. Nearly one in six deaths result from cancer, approximately ten million deaths and of these, around 4,000 were children (accounted by WHO in 2020) (10). According to 'Cancer Statistics, 2023' reported by the American Cancer Society (ACS), there will be more than 1.9 million new cancer cases and 0.6 million cancer deaths in the US. Besides, there will be about 89 thousand melanoma new cases (11).

In addition, lung cancer (LC) is the leading cause of cancer death worldwide in both genders (13). Approximately 2.2 million new cases were diagnosed worldwide with nearly 1.8 million deaths from LC in 2020 (13). ACS also estimated that Nearly 90% of American lung cancer cases occur in aged over 55 people (14). Lung cancer usually is divided into two main types: non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC) (15). And 80%-85% of LC are NSCLC. Smoking is a well-known risk factor for lung cancer. In addition, occupational exposure, air pollution, poor dietary habits, and genetic susceptibility also increase the incidence of lung cancer (16). Although the incidence of LC in some developed countries has been reduced because of the promotion of quitting smoking. However, developing countries, such as China, with high tobacco consumption still have an increasing trend in LC incidence(17). Therefore, finding a way to decrease the number of LC patients number is a necessity.

Although skin cancer accounts for only around 1% of all cancer deaths, its incidence has increased significantly since the 1970s, mainly due to lifestyle changes, including sun-seeking behavior, and the thinning of the ozone layer (18,19). Similarly, skin cancer also includes two types: cutaneous melanoma (CM) and nonmelanoma skin cancer (NMSC) (20). In addition, due to the less concentrated melanin and more exposure

behaviour, compared with other darker skin tones, white skin tone people are more likely to develop skin cancer (21). Despite Melanoma is less common, it has higher mortality rates because of its poorer prognosis - nearly 30 thousand new CM cases were diagnosed with 5.7 thousand deaths worldwide in 2020 (22). However, timely diagnosis and early treatment can significantly are closely associated with reducing mortality, which also avoids additional health problems and economic benefits (23). While the indistinguishability of early skin cancer from moles limits the early detection of skin cancer. Hence, it is crucial to find suitable markers for the detection of SC (24).

The types of cancer treatment depend on the type of and advance of cancer. Surgery is still the main therapy for solid tumours. However, surgery may not get rid of all cancers, such as those near very delicate tissues, those metastases to multiple other organs, or cancers of the blood system and lymphatic systems (25). Radiation therapy is a treatment that kills cancer cells with high doses of radiation, which still is a critical cancer treatment (26). When the tumour spreads, chemotherapy is recommended to relieve cancer-related symptoms (27). Besides, hormone therapy, biotherapy, immunotherapy, and targeted therapy are also useful cancer treatments. However, two or more combined treatment models are more often.

Due to the side effects of cancer treatment, precision medicine in oncology, a specific kind of treatment and care based on particular genes, proteins, immune environment and other substances, is emerging (28). And biomarker testing provides that information (29). Take the TP53 gene as an example, it is one of the most frequently mutated genes in cancers (30), which involves DNA repair, metabolism, and cell senescence and apoptosis (31). Hence, the TP53 gene has been certified as a tumour suppressor gene, including lung cancer and skin cancer (32). The long intergenic non-protein coding RNA TP53-induced transcript (LINC-PINT) is one of the TP53-induced transcripts and also a tumour suppressor, which is found in osteosarcoma (33), gastric cancer (34), renal cell carcinoma (35), glioblastoma (36), melanoma (37), and lung cancer(38) (including NSCLC (39). LincRNA-P21 is also a direct transcriptional target of TP53 (40), which directly or indirectly regulates cancer cell proliferation, migration and

apoptosis (41). Thus, LINC-PINT and lincRNA-P21 as new biomarkers in cancer are interesting.

Single nucleotide polymorphisms (SNPs), as a well-known biomarker, are also being investigated. for example, FokI, as a single-nucleotide polymorphism coded rs2228570 in the vitamin D receptor (VDR) gene, is related to many cancers prognosis, breast cancer (42), ovarian cancer (43), gastric cancer (44), hepatocellular carcinoma (45), papillary thyroid cancer (46), pancreatic cancer (47) and melanoma. Or, SNPs rs13181 and rs238406 locate in the ERCC2 gene, which involves in DNA repair functions. They are also identified in lung cancer (48), cervical cancer (49), breast cancer, squamous cell carcinomas of the head and neck (50), and bladder cancer (51).

However, many cancers can be prevented if controlled the risk factors, such as smoking, abusing alcohol, and being overweight. It even can be cured if detected, diagnosed and screened early and treated effectively (52).

1.3 How molecular epidemiology works in cancer research

1.3.1 Stages from exposure to diseases development

To explore the correlation between the cause (exposure) and the outcome (cancer), “traditional” epidemiology employs a series of studies from observation to experiment. However, no matter whether the descriptive, analytic, or experimental studies, “traditional” epidemiology revolves around time, place and person (53). Besides, “traditional” epidemiologic is more common to collect personal information, social information, environmental information, and disease-related information. Even when epidemiologists collect biometric information, it is mostly biological macromolecules (54). However, as shown in Figure 1 and mentioned before, molecular epidemiology pays attention to looking for biomarkers related to diseases, such as DNA, transcription factors, RNA, cell surface receptors, enzymes, and even metabolites, and then uses them to explain the diseases' mechanisms in populations (55). Among them, “biomarker” is a broad term, defined by WHO that “almost any measurement reflecting an

interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction." (56).

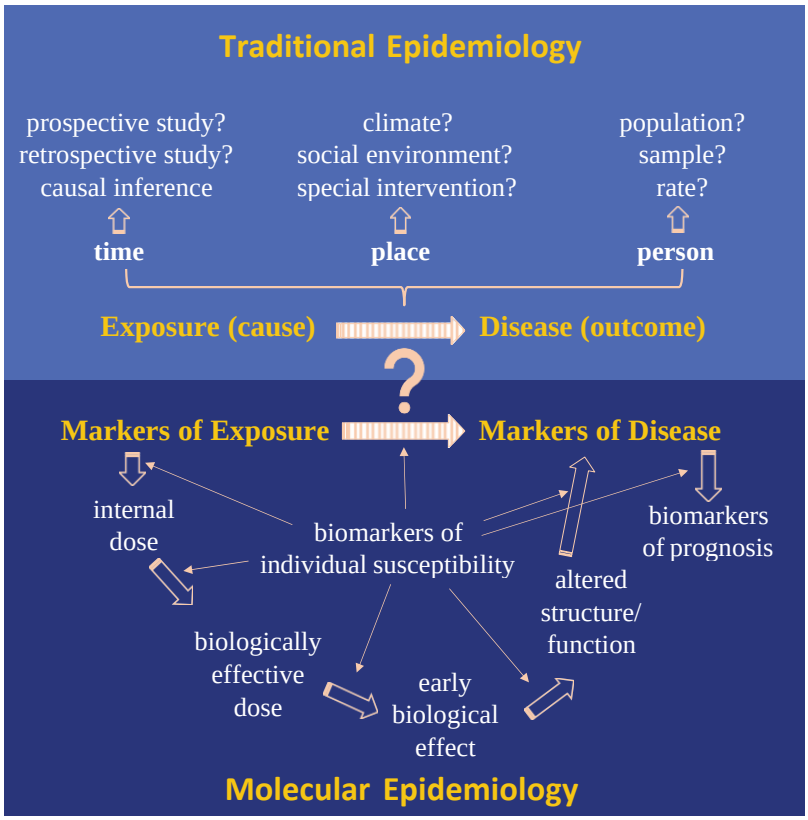


Figure 1 The difference between epidemiology and molecular epidemiology

1.3.2 Biomarkers of exposure

As shown in Figure 1, according to the stages from exposure to cancer development, biomarkers used in cancer molecular epidemiology research can be categorized into different classes. First of all, is biomarkers of exposure. Biomarkers of exposure are chemicals, their metabolites, or even the interactive products between the xenobiotic and organism, that can be measured in the body or biological sample, which reflects the levels and characteristics of exposure in the organism. In simple words, it is the substance directly or indirectly found in the body after people are directly or indirectly exposed to the chemicals. Usually, biomarkers of exposure are measured in the blood,

urine, saliva, and even hair. Many chemicals have the potential to be biomarkers of exposure, but an ideal exposure biomarker is considered to include the following criteria: corresponding to a special chemical, remaining long enough for measurement, and simply measuring.

Bisphenol A is a classic example of this. Bisphenol A, broadly used in plastics, is a xenoestrogen, which has hormone-like characteristics (57). Its metabolite, d16-Bisphenol A glucuronide, as a biomarker, can be detected in blood and urine samples, which reflects the exposure to bisphenol A (58). Biomarkers of exposure to cancer disease are also common. For example, inorganic arsenic exiting in food, water, and even air. It has been certified as associated with various internal cancers and skin cancer. However, arsenic exposure is easily ignored because it lacks colour, odour, and flavour. Hence, arsenic, as a biomarker for directly evaluating body arsenic exposure, is measured by blood, hair, and urine samples (59). Exposure biomarkers have been widely used in epidemiology.

1.3.3 Internal dose biomarkers

Then, internal dose biomarkers were produced from exogenous exposure markers. Due to their electrophile, certain externally acquired carcinogens or their metabolites can covalently bind with DNA or proteins in the nucleophilic sites to form DNA adducts (60). Carcinogen–DNA adducts may upregulate or silence gene expression patterns, or even cease the expression of some genes, and then bring about protein abnormality or absence that results in the uncontrolled growth of cells (cancer). For instance, environmental carcinogen dimethylbenz(a)anthracene (DMBA), as a tumour initiator widely used in cancer research, its DNA adducts specifically mutate Ha-ras, a proto-oncogene, into a carcinogen mutant form (61,62).

However, carcinogen-DNA adducts are instability and unnormal DNA also will be repaired. Hence, they are only internal dose biomarkers, otherwise, they will be called biologically effective dose markers (63). Take bladder cancer for example, smoking is

an independent risk factor for bladder cancer (64,65). Polycyclic aromatic hydrocarbons (PAHs), especially B[a]P, from the cigarette can form stable adducts with the exocyclic amino groups of purines of DNA or can form unstable adducts with the N7 or C8 of purines, after a series of metabolic transformations (65). Mutations may be caused by PAH–DNA adducts, which further inactivate tumour suppressor genes or activate proto-oncogenes (66). Adducts are not limited to DNA, and carcinogen–protein adducts also can be considered as a biomarker of the biologically effective dose. But only the DNA adduct leads to critical mutagenic changes (67).

1.3.4 Biomarkers of biological effect

Carcinogen-DNA adducts or their protein adducts also be recognized as biomarkers of early biological effect if they can cause mutations inducing cancer (68). Therefore, identifying and developing the biomarkers of early biological effects are the focus of research. A very famous example is the TP53 gene pathway. It is a complex network of genes, which involved in DNA repair, metabolism, cell cycle arrest, and cell senescence and apoptosis(69). Following the integrity of the single-stranded or double-stranded DNA is damaged by intrinsic or extrinsic stresses, a rangE of enzyme-relavant TP53 geneS are activated, such as kinases ataxia–telangiectasia mutated kinase (ATM) and ataxia-telangiectasia (ATR) (69). TP53 phosphorylated by enzymes is released from mouse double minute 2 homolog (MDM2) and in turn, acts as a transcription factor to induce the expression of various tumor suppressor genes (70,71).

Therefore, a variety of anticancer treatments targeting the mutant TP53 gene were developed including in breast cancer, lung cancer, ovarian cancer, and hematopoietic cancer (72). Among them, the treatment of lung cancer will be explained in detail in the later article. Biomarkers of early biological effects improve the accuracy of exposure assessment, such as the most widely used chromosomal aberration (CA) which is also the marker of altered structure /function (73). The ability to evaluate and prediction in cancer of CA has been confirmed in several malignancies, such as respiratory cancer, gastrointestinal cancer and genitourinary cancers (74).

1.3.5 Markers in cancer staging, treatment, and prognosis

Apart from early detection of the asymptomatic patients just mentioned before, biomarkers play important roles in cancer diagnosis, staging, treatment, and prognosis. A particular example in the diagnosis and staging area is to determine the origin of cancer: primary or metastatic. For example, the level of prostate-specific antigen (PSA) has been used as an indicator (over 100 ng/mL) for metastatic prostate cancer (75). Or mammaglobin and gross cystic disease fluid protein-15 (GCDFP-15) have been used as a marker in identifying breast cancer subtypes and metastatic.

Biomarkers are critical in the treatment of tumours(76–78). Restoring the wild-type activity of mutp53 is a novel therapeutic strategy. That is because the spatial conformation and folding pattern of mutp5 can be changed by small molecule compounds and peptide drugs, such as APR-246, SAHA, COTI-2, and PEITC (79). APR-246 is a pro-drug, which, after activating methylene quinuclidinone (MQ), can bind the TP53's critical cysteines, change the TP53's conformation, and then activate TP53 (80). Hence, APR-246 has been combined used with multiple drugs in the anti-cancer treatment of acute myeloid leukaemia, oesophagal cancer, and breast cancer (81).

Similarly, the prognosis of tumours is also inseparable from biomarkers that are detected from the organ, blood, saliva, or urine. Circulating tumour DNA (ctDNA) is a kind of single-stranded or double-stranded DNA in the plasma or serum that is released by the tumour cells (82). Apart from using it as an early bio-effective, diagnostic, staging and metastatic biomarker, it also involves in the tumour prognosis (83). It is well known that malignant tumour progression or recurrence is the hard point in treatment. However, it has been investigated that the concentration of DNA in the blood changes in accordance with the stage of tumour development (84). It increases with cancer progression or decreases after cancer surgery (85). Hence, blood ctDNA can be employed as a quick detection for clinicians in predicting therapy outcomes. Especially, for patients who need long-term follow-up (86).

1.4 How molecular epidemiology is employed in specific practical studies

However, as a novel field, how molecular epidemiology is used in specific practical studies, and how it helps to discover causes, explore risk factors, and ultimately protect, still remains an unclear explanation. Hence, the aim of this study is to display how molecular epidemiological runs in cancer research through two practical studies: 'The relationship between single nucleotide polymorphisms and skin cancer susceptibility' and 'The treatment effect of hydrogen gas on lung cancer'.

1.4.1 The example of protecting against cancer - Hydrogen gas affects the expression of LINC-PINT and Lin-cRNA-P21

Background: Lung cancer (LC), as one of the most common cancer, causes increased numbers of morbidity and mortality all over the world. The long intergenic non-protein coding RNA p53-induced transcript (LINC-PINT) and LincRNA-p21 are TP53-induced transcripts. It was investigated that the expression of LINC-PINT and lincRNA-P21 decreased in the malignant cancer. Molecular hydrogen (H_2) is a new medical gas that is used as a selective antioxidant in the anti-inflammation and anti-apoptosis functions modulating. H_2 also plays a role in the LC treatment. Therefore, the aim of this study is to explore the treatment effect of H_2 in lung cancer cells by identifying the expression of LINC-PINT and lincRNA-P21.

Method: Electrochemical water device was utilizted to produce hydrogen gas. qRT-PCR was employed to assess the expression of LINC-PINT and lincRNA-P21 in lung cancer cells, respectively. One-way analysis of variance (ANOVA) and linear regression were carried out for analysing multiple groups' differences and associations.

Result: The expression of LINC-PINT was significantly correlated with the increasing (from 0% to 5% to 10%) H2 concentrations, after both 2-hour-30-minute(2H30) and 3-hour(3H) hydro-gen treatment (R22H30=0.52, R23H=0.57, both $P<0.05$). This positive relationship -between expression and H2 concentrations- was also observed for lincRNA-P21 expression in the 2H30 group (R2=0.88, $P<0.01$). In contrast, the

expression of LINC-PINT showed a significant negative correlation with H2 after 3-hour-30-minute (3H30, R2=0.81, P<0.01). Besides, the expression of lincRNA-P21 decreased with time (F5%H2=13.54 and R25%H2=0.66, F10%H2=28.94 and R210%H2=0.81, all P<0.01) in both 5% and 10% H2 concentrations groups.

Conclusion: Our study indicated the expressions of LINC-PINT and lincRNA-P21 were upregulated after H2 gas treatment.

1.4.2 The example of cancer risk assessment - The relationship between single nucleotide polymorphisms and skin cancer susceptibility

Background: Risk assessment is the earliest evidence of impending cancer in persons who don't have cancer. Single nucleotide polymorphisms (SNPs) interfere with the function of certain genes and thus may influence the probability of skin cancer (SC). The correlation between SNPs and skin cancer lacks statistical power, however. Therefore, the purpose of this study was to identify the gene polymorphisms involved in skin cancer susceptibility using network meta-analysis, and to determine the relationship between SNPs and SC risk.

Methods: PubMed, Embase and Web of Science were searched for articles including ‘SNP’ and different types of SC as keywords, between January 2005 and May 2022. The Newcastle-Ottawa Scale was used to assess bias judgment. Odds ratio (ORs) and their 95% confidence intervals (CI) were determined to estimate heterogeneity within and between studies. Meta-analysis and network meta-analysis were carried out to identify the SNPs associated with SC. The P-score of each SNP was compared to obtain the rank of probability. Subgroup analyses were performed by cancer type.

Results: 275 SNPs from 59 studies were included in the study. Two subgroup SNP networks using the alleles and dominant models were analyzed. The minor alleles of rs2228570 (FokI) and rs13181(ERCC2) were the first-ranking SNPs in both subgroups one two in the alleles model, respectively. Wildtype, and heterozygous genotypes of rs475007 in subgroup one and the mutated homozygous genotype of rs238406 in

subgroup two were most likely to be associated with skin cancer based on the dominant model.

Conclusions: According to the alleles model, SNPs FokI rs2228570 and ERCC2 rs13181 and according to the dominant model, SNPs MMP1 rs475007 and ERCC2 rs238406 are closely linked to SC risk.

2 The therapeutic effect of hydrogen gas on lung cancer -- Linc-PINT and LincRNA-p21 as biomarkers

2.1 Introduction

Lung cancer (LC) is the second most common cancer in both genders, and it is the leading cause of cancer mortality globally (12). Approximately 2.2 million new LC cases were diagnosed worldwide with nearly 1.8 million deaths in 2020 (13). Lung cancer is caused by the uncontrolled cell growth in the tissues of the lung and is classified into two major histological types: small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC) (15). NSCLC accounts for 80%-85% of lung cancers. Although the main etiologic factor is tobacco use, other risk factors such as occupational exposures to hazardous chemicals, air pollution, poor diet and genetic susceptibility may also increase the risk of LC (16). Since smoking cessation policies were implemented, a downward trend has been reported in the incidence of LC in the developed countries. However, developing nations, such as China -where tobacco consumption has remined high- have not experienced a decline in LC incidence (17). With the introduction of low-dose CT screening, important developments in the timely diagnosis of LC have been made in recent years (87). However, to date, most lung cancers are diagnosed at an advanced stage, at stages 3 or 4, when the efficacy of treatment is more limited (88,89). LC continues to constitute a major health burden throughout the world, particularly in developing countries, therefore identifying new, effective options for the treatment of LC is crucial.

The TP53 gene is a widely researched tumor suppressor gene, located on chromosome 17. As one of the most frequently mutated genes in human cancers (30), coding the tumor protein p53, it regulates the expression of a range of genes from DNA repair, metabolism, cell cycle arrest to cell senescence and apoptosis (31). TP53 has been found to be associated with the development of various cancers, including lung cancer (32).

Long non-coding RNAs (lncRNA) are a kind of RNA with more than 200 nucleotides which are not translated into protein (90). With the development of genome-wide sequencing and high-resolution microarray technologies (91,92), lncRNAs have been shown to be linked to gene transcription, post-transcriptional regulation, epigenetic regulation, and DNA regulation (93).

The long intergenic non-protein coding RNA TP53-induced transcript (LINC-PINT) is a TP53-induced transcript, located on chromosome 7, spanning 232,616 bases (94). LINC-PINT is considered to be a promising lncRNA tumor suppressor gene. Decreased expression levels of LINC-PINT have been found in various cancers such as osteosarcoma (33), gastric cancer (34), renal cell carcinoma (35), glioblastoma (36), melanoma (37), and lung cancer (38), including NSCLC (39). Hence, LINC-PINT has been investigated as a possible marker for promoting tumor progression (95) and predicting prognosis (40).

LincRNA-P21 is also a direct transcriptional target of TP53 (40) and has been reported to play a part downstream of TP53-mediated transcriptional repression. By interacting with heterogeneous nuclear ribonucleoprotein K (hnRNP K), and TP53 upregulated modulator of apoptosis (PUMA), lincRNA-P21 regulates growth arrest and apoptosis (96,97). In addition, lincRNA-P21 also interacts with MDM2 to regulate TP53 levels (96). LincRNA-P21 has been found to directly or indirectly influence the proliferation, migration, apoptosis and the Warburg effect of cancer cells, by binding to different miRNAs and proteins (41). Thus, the possible role of lincRNA-P21 as a new biomarker in cancer has been suggested.

Molecular hydrogen (H_2) is a colorless, odorless, and flammable gas. It has been utilized as a novel medical gas since its possible therapeutic effects were first investigated in vivo, in mouse skin squamous carcinoma (98). H_2 has been reported to be a selective antioxidant, by decreasing the free radicals, ·OH and ONOO- in living cells (99). H_2 has also been found to interact with superoxide dismutase (SOD), adenosine triphosphate (ATP), nuclear factor erythroid 2-related factor 2 (Nrf2) and cytoplasmic heme oxygenase-1(HO-1) (100–102). Hence, the anti-inflammation and anti-apoptotic properties of H_2 have led to its increased investigation in anticancer research (103,104).

The aim of the present study was to explore the effect of H2 in lung cancer cells by determining the expression levels of LINC-PINT and lincRNA-P21.

2.2 Materials and methods

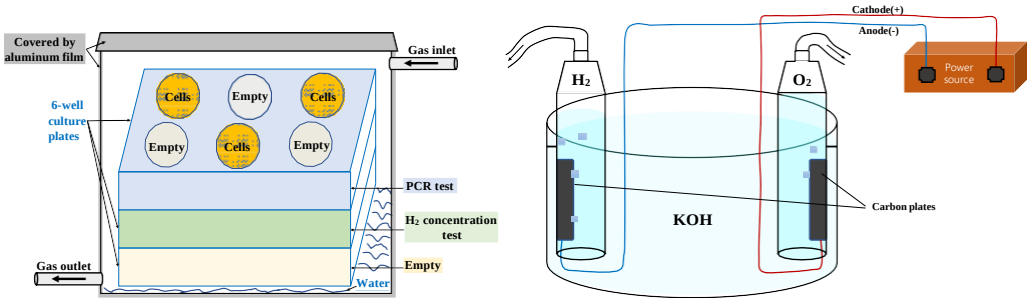
2.2.1 Cell culture

A549 cell lines were kindly gifted by Professor Kata Juhász at the Medical School, Univesity of Pécs. The A549 NSCLC cells were cultured in Dulbecco's MEM (DMEM) media containing 10% fetal bovine serum (FBS) and 100 IU/ml penicillin-streptomycin. Cells were then maintained in the incubator at 37 ℃ temperature and 5% CO2.

2.2.2 Treatment with H_2 gas

Cells were treated in 3 wells of 6-well culture dishes in closed plastic boxes with a volume of 1950-ml^3 (15cm*10cm*13cm). As shown in Figure 2(a), the sides and tops of the boxes were covered with aluminium to reduce the possibility of the escape of hydrogen gas (105). Water was added to the bottom of the boxes for maintaining humidity (106). Hydrogen gas was delivered into the box via a top afferent tube and out of the box via a bottom efferent tube. From the three layers of the 6-well culture dish, the top one was used for the PCR test, the middle one for the H_2 concentration test and the bottom one was left empty. H_2 concentration was tested by the H_2 meter (YIERYI,

4-in-1 water quality test meter, from 0 to 2.400ppm, with a 2ppm resolution, and ±10ppm accuracy). An electrolyzed water device was employed to produce H_2 gas (Figure 2(b)).



a b

Figure 2 The schematic diagram of cell culture with H_2 gas (a) and electrolyzed water device (b).

2.2.3 RNA isolution and quantitative Real-Time PCR

Total RNAs were isolated from lung cancer cells using the TRIzol method (Invitrogen, USA), and then air-dried and stored at -70°C. DNA oligos from Integrated DNA Technologies (IDT) (Coralville, IA, USA) were used as a template for quantitative real-time (RT)-PCR. The primers were the following:

forward: 5′AGGAGGGAACGAGGCAGGGA3′

and reverse: 5′AGCTCAGATCAGCAAGGCAG3′ for lincRNA-PINT

and forward: 5′GGGGATAAGCACCACTAATG3′

and reverse: 5′TGTAGGCAATCACAGAGCAC3′ for lincRNA-P21 (107).

The expression of RNAs was tested with the LightCycler- 480 Instrument II RT-PCR

System (Roche, Swiss). Melting temperatures (Tm) of the forward and reverse primers of lincRNA-PINT were 62.8 °C and 57.4 °C and those of lincRNA-p21 were 52.9 °C and 55.0 °C. The 2–ooCt method was employed for the assessment of the expression levels of lincRNA.

2.2.4 Statistical analysis

One-way analysis of variance (ANOVA) and linear regression were used to clarify multiple groups' differences and associations. We respectively delivered 0% (control group), 5% H_2 gas (5%H_2 group) and 10% H_2 gas (10%H_2 group) into the lung cancer cells for two hours and thirty minutes (2H30 group), three hours group (3H group), and three hours and forty minutes group (3H40 group). $P<0.05$ was defined as the statistical significance and $P<0.01$ was described as a significant statistical difference. Analyses were carried out by IBM SPSS version 26.0.

2.3 Results

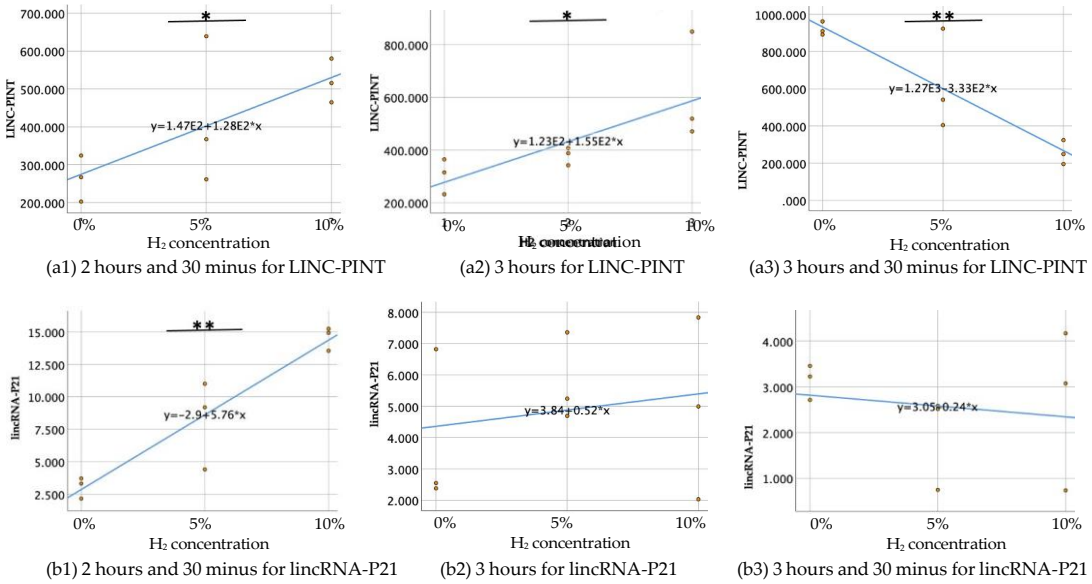
2.3.1 Investigation of possible influencing factors

To ensure that flow of H_2 gas was stable, we investigated the relationship between H_2 and time. The volume of the produced hydrogen had a linear relationship with time ($Y=32.78+1.55X$, $R^2=97.60\%$, $P<0.05$). Besides, ANOVA result showed that there was no statistical difference between the H_2 concentration of the three layers of the box ($F=0.589$, $P>0.05$). Thus, we confirmed, that the middle and the top layers of the culture dish were exposed to equal concentrations of hydrogen gas.

2.3.2 Effect of H_2 concentration on lincRNAs expression

Lung cancer cells were treated with 3 different concentrations (0%,5%,and 10%) of H_2 gasfor three different time periods (2H, 3H, and 3H30). As shown in Figures 2(a1) and (a2), the expression levels of LINC-PINT were significantly correlated with the increasing H_2 concentrations, from 0% (control group) to the 5% and 10%-

concentrations in the 2H30 and 3H time groups (R^2_{2H30}=0.52, R^2_{3H}=0.57, both P<0.05). The positive relationship between expression levels and H_2 concentrations was also observed for lincRNA-P21 expression in the 2H30 time group (R^2=0.88, P<0.01). In contrast, the expression of LINC-PINT showed a significant negative correlation with H_2 concentration in the 3H40 time group (R^2=0.81, P<0.01).



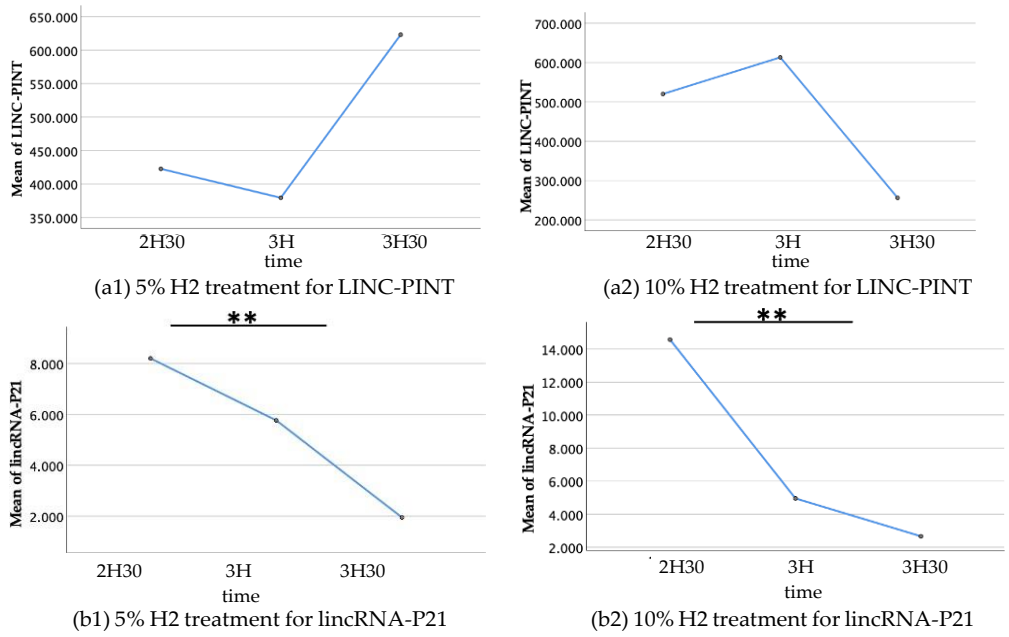
(a1) 2 hours and 30 minus for LINC-PINT (a2) 3 hours for LINC-PINT (a3) 3 hours and 30 minus for LINC-PINT

(b1) 2 hours and 30 minus for lincRNA-P21 (b2) 3 hours for lincRNA-P21 (b3) 3 hours and 30 minus for lincRNA-P21

Figure 3 The relationship between the expression of LINC-PINT (a) and lincRNA-P21 (b) and H_2 concentration evaluated after differenttime periods. (a1) 2 hours and 30 minus for LINC-PINT, R^2 linear = 0.52; (a2) 3 hours for LINC-PINT, R^2 linear = 0.57; (a3) 3 hours and 30 minus for LINC-PINT, R^2 linear = 0.81; (b1) 2 hours and 30 minus for lincRNA-P21, R^2 linear = 0.88; (b2) 3 hours for lincRNA-P21, R^2 linear = 0.03; (b3) 3 hours and 30 minus for lincRNA-P21, R^2 linear = 0.03; For the x-axis: 1=0% H_2 (control group), 2=5% H_2, and 3=10% H_2; Y represents the relative concentration levels (subtract the concentration of the reference gene-GAPDH) of LINC-PINT (a) and lincRNA-P21 (b); "*" indicates statistical difference (P<0.05) and "**" indicates significantly statistical difference (P<0.01).

2.3.3 Effect of treatment time on linc-RNA expression

The expression levels of LINC-PINT in the 5% H_2 and the 10% H_2 group followed opposite trends after different lengths of treatment, however neither trend was statistically significant ($F_{5\%H2}$=1.60 and $R^2_{5\%H2}$=0.78, $F_{10\%H2}$=3.64 and $R^2_{10\%H2}$=0.34, all P>0.05) (Figure 3(a1) and (a2) of Supplement). The expression of lincRNA-P21 decreased with time ($F_{5\%H2}$=13.54 and $R^2_{5\%H2}$=0.66, $F_{10\%H2}$=28.94 and $R^2_{10\%H2}$=0.81, all P<0.01) in both 5% and 10% H_2 concentrations groups (Figure 3(b1) and (b2) of Supplement).



(a1) 5% H2 treatment for LINC-PINT

(a2) 10% H2 treatment for LINC-PINT

(b1) 5% H2 treatment for lincRNA-P21

(b2) 10% H2 treatment for lincRNA-P21

Figure 4 Expression of LINC-PINT (a) and lincRNA-P21 (b) in lung cancer cells after treatment with two H_2 concentrations for different time periods. (a1) 5% H_2 treatment for LINC-PINT; (a2) 10% H_2 treatment for LINC-PINT; (b1) 5% H_2 treatment for lincRNA-P21; (b2) 10% H_2 treatment for lincRNA-P21; Y-axis represents the mean of relative concentration levels (subtract the concentration of the reference gene-GAPDH) of LINC-PINT (a) and lincRNA-P21 (b); "*" indicates statistical difference (P<0.05) and "**" indicates significantly statistical difference (P<0.01).

2.4 Discussion

To our knowledge, this is the first study to identify the possible role of lncRNAs as biomarkers in lung cancer cells after hydrogen gas useage. Our results indicated that hydrogen not only influenced the cells' functions at the DNA and protein levels, but also affected their lncRNA expression. Furthermore, we found that LINC-PINT expression increased with increasing concentrations of H_2 gas, after 2 hours thirty minutes and three hours. Furthermore, similar to LINC-PINT, lincRNA-P21 expression was found to be lowest after using 5% H_2 gas, followed by the 10% , then 0% H_2, with period of 2 hours and 30 minutes.

The TP53-induced transcript, LINC-PINT has been detected in multiple types of human tissue (108). LINC-PINT negatively modulates TP53 in an autoregulative manner by acting as a regulator of cell cycle arrest and a pro-survival molecule when DNA damage occurs. LincRNA-P21 is also a tumor suppressor (109), which has been shown to competitively bind to Mouse double minute 2 (MDM2), to increase the transcriptional activity of TP53 (110). Thus, LINC-PINT and lincRNA-P21 directly and indirectly, regulate cell proliferation, migration, apoptosis, and the Warburg effect (41) and they are also essential for cell growth and proliferation (111).

Hence, our findings indicate that H2 gas upregulated the expression of LINC-PINT and lincRNA-P21 in lung cancer cells, which was also reported previously (112). For example, a patient-based study involving LC patients indicated that, compared to normal tissue, the expression of lincRNA-P21 was decreased in tumor tissue (113). This downregulation of lincRNA-P21 in NSCL as also be described in Samaneh Talebi's study (114). Dongchang Wang et al reported that treatment with H2 gas inhibited the growth, migration, invasion, and apoptosis of A549 and H1975 cells by down-regulating a regulator for chromosome condensation (115).

Interestingly, however, we found that in the cells treated with H_2 gas for the longest period of time (3 hours and 40 minutes), the expression levels of LINC-PINT decreased.

Our investigation also demonstrated that the expression of lincRNA-P21 decreased with time in the 5% and 10% H_2 treatment groups. Our findings are supported by Castellano et al. Their sutdy reported that lung cancer patients with a worse prognosis had higher lincRNA-P21 levels than those with a better prognosis (113).

LincRNA-p21 has been shown to be a hypoxia-responsive lncRNA that plays an important role in glycolysis by binding to HIF-1α and VHL under hypoxic circumstances (116). The Warburg effect can be defined as a form of disrupted glucose metabolism, with an increased rate of glucose consumption and production of lactate despite the presence of oxygen, which is typical for tumors and malignant evolution (117). This metabolic characteristic the Warburg effect has also been shown to contribute to the invasion and metastasis of lung cancer malignancies (118,119). In previous studies, LINC-PINT has been found to be negatively correlated to HIF-1α, an oxygen-sensing transcription factor, in gastric cancer cells (120). Thus, it is highly probable that LINC-PINT and lincRNA-P21 are also involved in the disruption of glucose metabolism in lung cancer cells (121). Based on these data, therefore, we hypothesize that treatment with hydrogen may have induced the Warburg effect in lung cancer cells, which in turn resulted in the decreased expression of LINC-PINT and lincRNA-P21. Further research is warranted, however, to verify our hypothesis.

2.5 Conclusions

In summary -although LINC-PINT and lincRNA-P21 levels decreased in the relatively long H_2 groups- our study indicated that the expressions of LINC-PINT and lincRNA-P21 were upregulated with increasing concentrations of H_2 gas after both two hours and thirty minutes and three hours of treatment.

Thus, it can be concluded that hydrogen gas upregulated the expression of LINC-PINT and lincRNA-P21 in non-small cell lung cancer cells after a comparatively short usage period.

2.6 Limitation

Although each experiment was repeated three times, the number of repetitions may still constitute a main limitation of this study. Secondly, the H_2 treatment time ranged between 2 hours 30 minutes and 3 hours 40 minutes, which cannot be considered long time intervals. Thus, our results may have differed if longer treatment times had been applied.

3 The relationship between single nucleotide polymorphisms and skin cancer susceptibility: A Systematic Review and Network Meta-Analysis

3.1 Introduction

The incidence of skin cancers has increased significantly since the 1970s, mainly due to lifestyle changes, including sun-seeking behavior, and the thinning of the ozone layer (18). Skin cancers include cutaneous melanoma (CM) and nonmelanoma skin cancer (NMSC), with growing incidence rates of both cancer types (20). They are all caused by the abnormal growth of skin cells, especially of those exposed to the sun. Non-melanoma skin cancer is the most common cancer among white-skinned people, thus it is a significant cause of morbidity (21). Melanoma is less common, however its prognosis is poorer resulting in higher mortality rates (22). Approximately 1.2 million new NMSC (22) and, nearly 300 000 new CM cases were diagnosed worldwide with 57 043 deaths from CM in 2020 (22). Timely diagnosis is crucial for reducing mortality from skin cancer and also has additional health and economic benefits (23). Since early detection of skin cancer is often limited, identifying suitable markers for its detection is of the utmost importance. Therefore, certain, new genetic loci were investigated as possible markers for identifying SC risk (24).

Single nucleotide polymorphisms (SNPs) are genetic variations caused by point mutations. The allelic distribution of SNPs may interfere with the function of genes and

then influence the probability of certain diseases (122,123), which has led to SNPs being investigated as possible biological markers. Various SNPs have been shown to be associated with pigmentation, nevi, hair, and skin color, melanin, and SC. The SNPs of the BRAF and NARS genes for example have been found to be commonly mutated oncogenes in CM (124). Furthermore, -similarly to the interactions between genetics and the environment- the number and frequency of SNPs also affect the characteristics of its related gene as well as the development of its related certain diseases phenotypes (125,126).

The network meta-analysis (NMA), and in particular, the Bayesian network meta-analysis, analyzes the direct and indirect evidence from multiple comparisons of tests within and between studies (127), making it possible to investigate the interactions between multiple comparisons of SNP tests.

Therefore, the aim of our study was to identify and compare the single nucleotide polymorphisms predominantly involved in skin cancer susceptibility by conducting a network meta-analysis.

3.2 Method

3.2.1 Search strategy

We searched the PubMed, Embase and Web of Science electronic databases from their starting date to May 2022, to identify relevant studies. The search strategy was shown in Presentation 1 of online supplementary file from website (https://www.frontiersin.org/articles/10.3389/fonc.2023.1094309/full#supplementary-material). We required the articles to include the following keywords: case-control, single nucleotide polymorphism (SNP), and study skin cancer (SC), cutaneous melanoma (CM), non-melanoma (NM), squamous cell carcinoma (SCC), or basal cell carcinoma (BCC). Inclusion and exclusion criteria are presented in Presentation 2 of online supplementary file. The study was designed and performed in accordance with the PRISMA guidelines (Figure 2).

3.2.2 Inclusion and exclusion criteria

Inclusion criteria were as follows: (1) study type is case-control trial (including Nested case-control study); (2) case group is the patients diagnosed with SC, CM, NM, SCC, and/or BCC; (3) control group is the non-such cancer healthy population; (4) genotypic detection method is PCR or DNA sequencing, such as PCR-based restriction fragment length polymorphism (PCR-RFLP), TaqMan PCR, Real-Time PCR (RT-PCR), Kompetitive allele specific PCR (KASP PCR), PCR with sequence-specific primers (SSP-PCR), Taqman, Cycle Sequencing Kit, and SNaPshot Sequencing Kit.

Articles were excluded based on the criteria: (1) cohort study, case reports, reviews, meeting abstracts, or comments; (2) uveal melanomas, non-skin squamous cell carcinoma (such as esophageal, neck, oral squamous cell carcinoma); (3) irrelevant or family cancer case group or unhealthy population control group; (4) genotypic detection method is not PCR or DNA sequencing (in particular, using microarray detection Genome-Wide Association Studies (GWAS); (5) lack of available genotype frequency; (6) duplicated articles or data.

It is important to point out that, for the fifth exclusion criterion, due to the same population (cases were melanoma patients at the Melanoma and Sarcoma Surgery Unit of the Istituto Nazionale Tumori, Milan, from May 2006 to June 2007. And controls were healthy donors from the Immunohematology and Transfusion Medicine Department, Fondazione IRCCS Istituto Nazionale Tumori), the same SNP (rs2910164), and the same conclusion (rs2910164 is a risk factor for melanoma), but the case/control number of study of Sangalli A(128) (304/314) is larger than that of Gomez-Lira M(129) (224/264). So we excluded Gomez-Lira M 's study, named ‘Association of microRNA 146a polymorphism rs2910164 and the risk of melanoma in an Italian population’. In the same manner, we excluded the 'Genetic Variants of the Vitamin D Receptor Gene Alter Risk of Cutaneous Melanoma' article(130) and only kept 'Haplotype and genotypes of the VDR gene and cutaneous melanoma risk in non-Hispanic whites in Texas: A case-control study' article(131).

3.2.3 Data abstraction and bias assessment

Two researchers (LZ and YS) – independently - screened the titles and abstracts of the search results and extracted the following information from the included articles: authors' name, year of publication, population of country and ethnicity, genotyping method, case and control numbers, control's source, case-control match, cancer type, gene, SNP, and allele frequency.

Subsequently we applied the Newcastle-Ottawa Scale (NOS) score for case-control studies to evaluate the quality and risk of bias of the included studies (Figure 3) (132). According to the NOS, article quality is assessed through eight questions from the Selection dimension (Cases definition, Cases selection, Controls definition, and Controls selection), the Comparability dimension (Comparability of cases and controls), and the Exposure dimension (Exposure ascertainment, Cases and controls ascertainment, and Non-response rate). Excepting 'Comparability' with two stars, other items can each be given one star. Hence, a study can be awarded a maximum of nine stars and will be excluded if lower than five stars. Discrepancies were resolved by consensus between the reviewing authors.

3.2.4 Data synthesis and statistical analysis

Alleles are represented differently in different genomes. Therefore, for clarity, all major alleles were represented by 'A' in this study, and the corresponding minor alleles were represented by 'B'. The allele model (A vs. B) was employed for exploring dominance. Furthermore, the dominant model (AA+AB vs. BB) and the recessive model (AA vs. AB+BB) were used for investigating the association between different genotypes and phenotypes (133).

A bivariate random effect model was performed for the meta-analysis of the comparative studies. Odds ratios (ORs) and their 95% confidence intervals (CI) were used for estimating heterogeneity within and between studies. Then, pooled sensitivity (Se), specificity (Sp), positive likelihood ratio (LR), negative likelihood ratio (LR),

diagnostic OR (DOR) and area under the summary receiver operating characteristic curve (AUROC) were calculated for each genotyping. The pooled AUROC was used as an indicator testing to examine the diagnostic accuracy of each genotyping. Statistical heterogeneity between each study was assessed using the inconsistency index I-square. Additionally, meta-regression analysis was performed based on cancer type to assess the heterogeneity. Cancer types included: CM, NMSC, and SC (including both CM and NMSC).

Next, the Bayesian network meta-analysis (NMA) was used to clarify the relationships between the SNPs and skin cancer according to the allele model (A vs. B) and the dominant model (AA+AB vs. BB). The Fixed-effects model that had four chains, 1000 burn-ins, 200 000 iterations, and a thinning interval of 10 was selected for the MCMC simulation (134). The Gelman-Rubin plot and Potential Scale Reduction Factor (PSRF) were used for assessing convergence. Net splitting was carried out for checking the consistency of the networks and the effect estimate table was employed for estimating all SNP comparisons. Then, the overall ranks of SNPs were estimated by P-scores that were equivalent to the surface under the cumulative ranking curve (SUCRA) (135). SNPs with the highest P-scores was considered to be the most related to skin cancer.

$P<0.05$ was considered to be statistically significant. RStudio software and StataSE 16.0 software were used for calculations and plotting. The software packages used in the study are are listed in Presentation 2 of online supplementary file.

3.3 Results

3.3.1 Literature search results

The literature search initially identified 3,575 studies from PubMed, Embase, and Web of Science. The search was ended on 2nd May, 2022. As Figure 7 shows, we screened 368 studies based on titles and abstracts and 232 full-text manuscripts. 59 studies met the inclusion criteria and were included in our network meta-analysis. One article was excluded due to bias, as explained below.

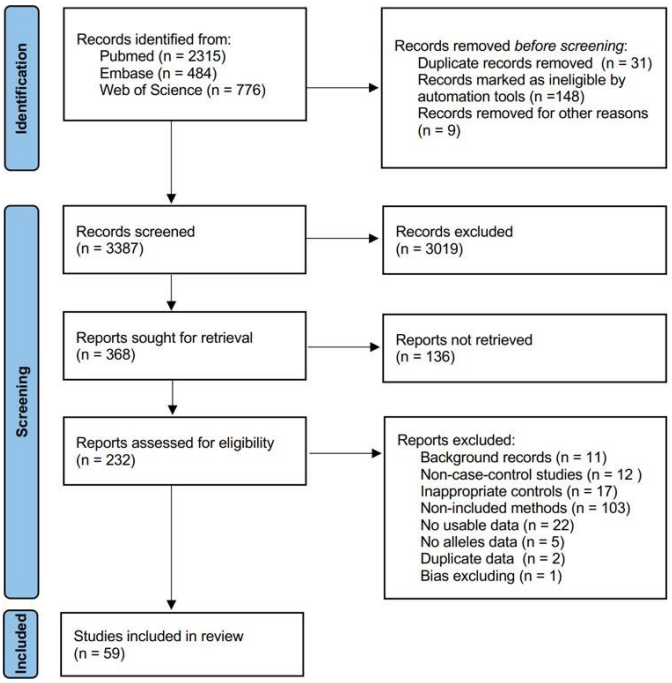


Figure 5 The literature search process and the screening flow chart for network meta-analysis.

3.3.2 Characteristics and Bias of Enrolled Studies

Table 1 summarizes the main characteristics of 60 studies, which were published between 2005 and 2022. Studies investigating Caucasian or Mongoloid ethnicities were included. Figure 8 shows the quality assessment of enrolled studies using the NOS risk bias tool. Any studies with NOS scores lower than five stars were excluded. Finally, there were 59 articles included in the systematic review and meta-analysis.

Table 1 Main characteristics of the eligible studies

Author	Year	Country	Ethnicity	Genotyping method	Case/ Control	Control's Source	Match
Jannot A-S(136)	2005	French	Caucasians	SNaPshot	120/125	HB	N
Vogel U(137)	2005	Denmark	Caucasian	RT-PCR	322/322	PB	Y
Li C (1)(138)	2006	USA	Caucasian	PCR	602/603	HB	Y
Li C (2)(139)	2006	USA	Caucasian	PCR	602/603	HB	Y
Wilkening S(140)	2007	Hungary,	Caucasian	TaqMan	517/523	HB	Y

		Romania and Slovakia					
Meyer P(141)	2007	Germany	Caucasian	Sequencing Kit	632/615	HB	N
Applebaum KM(142)	2007	USA	Caucasian	Taqman	1540/780	PB	Y
Povey JE(143)	2007	UK	Caucasian	PCR-RFLP	596/441	PB	Y
Pjanova D(144)	2007	Latvia	Caucasian	Sequencing Kit	203/125	HB	N
Li C(145)	2007	USA	Caucasian	PCR	602/603	HB	Y
Li C(131)	2008	USA	Caucasian	PCR	805/841	HB	Y
Fernandez LP(146)	2008	Spain	Caucasian	PCR	131/245	HB	Y
Guedj M(147)	2008	France	Caucasian	PCR	1019/1466	HB	N
Nan H(148)	2009	USA	Caucasian	PCR-RFLP	805/873	HB	Y
Schoof N(149)	2009	Germany	Caucasian	TaqMan PCR	165/162	HB	Y
Figl A(150)	2010	Germany and Spain	Caucasian	TaqMan	1186/1280	HB	Y
Capasso M(151)	2010	Italy	Caucasian	PCR	249/291	HB	N
Debniak T(152)	2011	Poland	Caucasian	Taqman	300/300	PB	N
Rizzato C (1)(153)	2011	Hungary, Romania and Slovakia	Caucasian	RT-PCR	507/515	HB	N
Rizzato C (2)(154)	2011	Hungary, Romania and Slovakia	Caucasian	Taqman	529/532	HB	Y
Lesiak A(155)	2011	Poland	Caucasian	PCR-RFLP	142/142	HB	Y
Wang L-E(156)	2011	USA	Caucasian	TaqMan	872/873	HB	Y
Almquist LM(157)	2011	USA	Caucasian	PCR-RFLP	1578/812	HB	Y
Ibarrola-Villava M(158)	2012	Spain	Caucasian	TaqMan PCR	562/338	HB	N
Helsing P(159)	2012	Norway	Caucasian	Sequencing Kit	388/420	HB	N
Santonocito C(160)	2012	Italy	Caucasian	RT-PCR	167/186	PB	Y
Cocos R(161)	2012	Romania	Caucasian	PCR-RFLP	174/80	HB	N
Gao R(162)	2013	USA	Caucasian	PCR	312/216	HB	N
Oliveira C(163)	2013	Brazil	Caucasian	PCR	146/146	HB	Y
Maccioni L (2)(164)	2013	Spain	Caucasian	PCR	837/1154	HB	Y
Pena-Chilet M (1)(165)	2013	Spain	Caucasian	RT-PCR	538/345	HB	N

Pena-Chilet M (2)(166)	2013	Spain	Caucasian	RT-PCR	530/314	HB	Y
Maccioni L (1)(167)	2013	Spain	Caucasian	PCR	837/1154	HB	Y
Francisco G(168)	2013	Brazil	Caucasian	PCR-RFLP	202/210	HB	Y
Yamashita J(169)	2013	Japan	Mongoloid	PCR	50/107	HB	N
Cordoba-Lanus E(170)	2014	Spain	Caucasian	Sequencing Kit	509/491	PB	Y
Gomez-Lira M(171)	2014	Italy	Caucasian	PCR-RFLP	240/342	HB	N
Oliveira C(172)	2014	Brazil	Caucasian	PCR	100/108	HB	Y
Thunell LK(173)	2014	Sweden	Caucasian	PCR-RFLP	50/799	PB	Y
Llorca-Cardenosa MJ(174)	2014	Spain	Caucasian	KASP PCR	648/381	HB	N
Hsu L-I(175)	2015	China	Mongoloid	PCR-RFLP	70/210	PB	Y
Russo I(176)	2016	Italy	Caucasian	RT-PCR	177/158	HB	N
Elefanti L(177)	2016	Italy	Caucasian	TaqMan	182/89	HB	N
Mukhammadiyeva GF(178)	2017	Russia	Caucasian	PCR-RFLP	25/100	PB	Y
Li Y-L(179)	2017	China	Mongoloid	TaqMan	660/662	HB	Y
Burns EM(180)	2017	USA	Caucasian	PCR	97/100	HB	N
Sangalli A(128)	2017	Italy	Caucasian	PCR	304/314	HB	N
Motorina AV(181)	2018	Russia	Caucasian	TaqMan PCR	95/334	PB	N
Gomez GVB(182)	2018	Brazil	Caucasian	RT-PCR	250/250	HB	N
Yuan T-A(183)	2018	USA	Caucasian	PCR	177/172	PB	N
Slawinska M(184)	2019	Poland	Caucasian	PCR	254/254	HB	Y
Orlandi E(185)	2019	Italy	Caucasian	PCR-RFLP	334/291	HB	Y
Ozola A(186)	2019	Latvia	Caucasian	RT-PCR	253/200	HB	N
Fathi F(187)	2019	Iranian	Caucasian	PCR-RFLP	210/320	PB	Y
Reis LB(188)	2020	Brazil	Caucasian	RT-PCR	120/135	HB	Y
Morgado-Aguila C(189)	2020	Spain	Caucasian	Taqman	81/73	PB	Y
Tovar-Parra JD(190)	2020	Colombia	Caucasian	PCR	85/170	HB	Y
Fathi F(191)	2021	Iranian	Caucasian	PCR-RFLP	210/220	PB	Y
Aristizabal-Pachon A (192)	2022	Colombia	Caucasians	PCR-RFLP	120/120	HB	Y
Dunjic M(193)*	2022	Serbian	Caucasians	RT-PCR	93/95	UN	Y

PCR: polvmerase chain reaction; PCR-RFLP: restriction fragment length polymorphism assay PCR; RT-PCR: real time PCR; KASP PCR: Kompetitive allele

specific PCR; PB: population-based; HB: hospital-based; UN: unknown; Y: yes; N: no; * Due to the low quality caused by bias, Dunjic M's article was excluded from the meta-analysis.

Low Risk of Bias (with starts)	
High Risk of Bias (without starts)	

Author	Year	Is the case definition adequate?	Representativeness of the cases	Selection of controls	Definition of controls	Comparability of cases and controls on the basis of the design or analysis (2 starts)	Ascertainment of exposure	Same method of ascertainment for cases and controls	Non-response rate	Total starts (9 starts)	Author	Year	Is the case definition adequate?	Representativeness of the cases	Selection of controls	Definition of controls	Comparability of cases and controls on the basis of the design or analysis (2 starts)	Ascertainment of exposure	Same method of ascertainment for cases and controls	Non-response rate	Total starts (9 starts)
Vogel U	2005	1	1	1	1	2	1	1	0	8	Maccioni L (2)	2013	1	1	0	1	2	1	1	0	7
Jannot A-S	2005	1	1	0	1	0	1	1	0	5	Gao R	2013	1	1	0	1	0	1	1	0	5
Li C (1)	2006	1	1	0	1	2	1	1	1	8	Oliveira C	2013	1	1	0	1	2	1	1	1	8
Li C (2)	2006	1	1	0	1	2	1	1	1	8	Francisco G	2013	1	1	0	1	2	1	1	0	7
Applebaum KM	2007	1	1	1	0	2	1	1	0	7	Yamashita J	2013	1	0	0	1	0	1	1	1	5
Povey JE	2007	1	1	1	1	2	1	1	0	8	Cordoba-Lanus E	2014	1	1	1	1	2	1	1	1	9
Wilkening S	2007	1	1	0	1	2	1	1	0	7	Thunell LK	2014	1	1	1	1	0	1	1	1	7
Meyer P	2007	1	1	0	1	0	1	1	0	5	Llorca-Cardenosa MJ	2014	1	1	0	1	0	1	1	0	5
Pjanova D	2007	1	1	0	0	0	1	1	1	5	Gomez-Lira M	2014	1	1	0	1	0	1	1	1	6
Li C	2007	1	1	0	1	2	1	1	1	8	Oliveira C	2014	1	1	0	1	2	1	1	1	8
Guedj M	2008	1	1	0	1	0	1	1	0	5	Hsu L-I	2015	1	1	1	1	2	1	1	0	8
Fernandez LP	2008	1	1	0	1	2	1	1	1	8	Russo I	2016	1	1	0	1	0	1	1	1	6
Li C	2008	1	1	0	1	2	1	1	1	8	Elefanti L	2016	1	1	0	1	0	1	1	0	5
Nan H	2009	1	1	0	1	2	1	1	0	7	Mukhammadiyeva GF	2017	1	1	1	1	2	1	1	1	9
Schoof N	2009	1	1	0	1	2	1	1	0	7	Li Y-L	2017	1	1	0	1	2	1	1	1	8
Figl A	2010	1	1	0	1	0	1	1	0	5	Burns EM	2017	1	1	0	1	0	1	1	0	5
Capasso M	2010	1	1	0	1	0	1	1	0	5	Sangalli A	2017	1	1	0	1	0	1	1	0	5
Debniak T	2011	1	1	1	1	0	1	1	0	6	Motorina AV	2018	1	1	1	1	0	1	1	0	6
Rizzato C (1)	2011	1	1	0	1	0	1	1	0	5	Gomez GVB	2018	1	1	0	0	0	1	1	1	5
Rizzato C (2)	2011	1	1	0	1	2	1	1	0	7	Yuan T-A	2018	1	1	1	1	0	1	1	0	6
Cocos R	2011	1	1	0	1	0	1	1	1	6	Fathi F	2019	1	1	1	1	2	1	1	1	9
Lesiak A	2011	1	1	0	1	2	1	1	1	8	Ozola A	2019	1	1	0	1	0	1	1	0	5
Wang L-E	2011	1	1	0	1	2	1	1	0	7	Slawinska M	2019	1	1	0	1	2	1	1	0	7
Almquist LM	2011	1	1	0	1	2	1	1	0	7	Orlandi E	2019	1	1	0	1	2	1	1	1	8
Ibarrola-Villava M	2012	1	1	0	1	0	1	1	0	5	Reis LB	2020	1	1	0	1	0	1	1	1	6
Helsing P	2012	1	1	0	0	0	1	1	1	5	Morgado-Aguila C	2020	1	1	1	1	2	1	1	1	9
Santonocito C	2012	1	1	1	1	2	1	1	1	9	Tovar-Parra JD	2020	1	1	0	1	2	1	1	1	8
Maccioni L (1)	2013	1	1	0	1	2	1	1	0	7	Fathi F	2021	1	1	1	1	2	1	1	1	9
Pena-Chilet M (1)	2013	1	1	0	1	0	1	1	0	5	Aristizabal-Pachon A	2022	1	1	0	1	2	1	1	1	8
Pena-Chilet M (2)	2013	1	1	0	1	2	1	1	0	7	Dunjic M	2022	0	0	0	0	2	0	0	0	2

Figure 6 Case-control risk of bias assessment graph.

3.3.3 Pairwise meta-analysis

A direct meta-analysis was performed to determine the correlation between 275 SNPs and SC risk (Table 1 of online supplementary file). 72 SNPs from 47 studies were closely associated with SC in the studies using the alleles model (A vs. B), while a significant association was found for 52 SNPs from 31 studies using the dominant

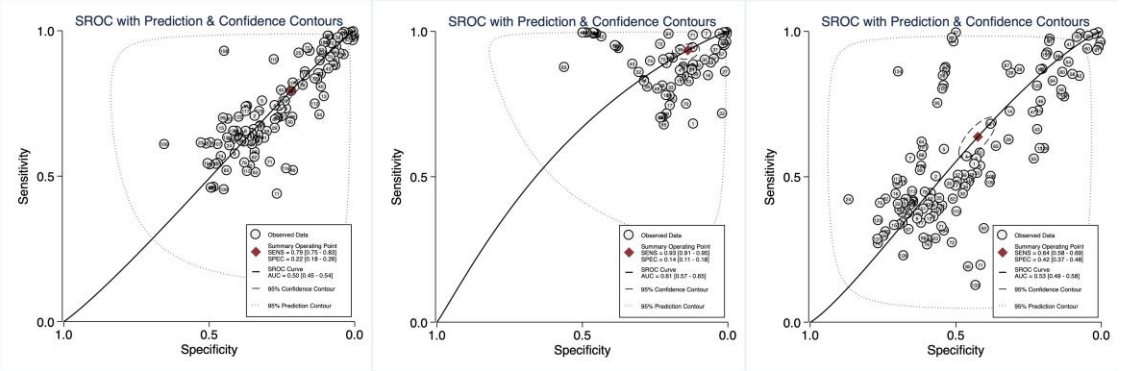
model (AA+AB vs. BB). Furthermore, based on the recessive model (AA vs. AB+BB), 77 SNPs from 35 studies were related to SC. As depicted in Table 1 of online supplementary file, the detected SNPs were analyzed further for diagnostic accuracy.

Table 2 shows the evaluation of the diagnostic performance of the pooled SNPs for SC. According to the SUCRA (Figure 9), the allele model can be employed for exploring dominance. Then, we chose the dominant model as the genotyping model for diagnosing SC.

Table 2 SNPs' diagnostic performance evaluation in the skin cancer

	Alleles model (A vs. B)	Dominant model (AA+AB vs. BB)	Recessive model (AA vs. AB+BB)
Number of studies	47	31	35
Number of SNPs	72	52	77
Pretest Prob	0.48	0.46	0.48
AUROC	0.50 [0.45, 0.54]	0.61 [0.57, 0.65]	0.53 [0.49, 0.57]
Sensitivity	0.79 [0.75, 0.83]	0.93 [0.91, 0.95]	0.64 [0.58, 0.69]
Specificity	0.22 [0.19, 0.26]	0.14 [0.11, 0.18]	0.42 [0.37, 0.47]
Positive LR	1.0 [1.0, 1.0]	1.1 [1.0, 1.1]	1.1 [1.0, 1.2]
Negative LR	0.94 [0.87, 1.02]	0.48 [0.33, 0.70]	0.85 [0.77,0.95]
Diagnostic OR	1 [1, 1]	2 [2, 3]	1 [1, 2]

'A' stands for the major alleles; 'B' stands the minor alleles; the numbers inside the '[,]' mean the range of 95% CI.



a. ROC curve of the alleles model b. ROC curve of the dominant model c. ROC curve of the recessive model

Figure 7 The ROC curve of three models.

3.3.4 The allele model (A vs. B)

The associations between the 72 SNPs and SC susceptibility are shown in Table 2 of online supplementary file. In the allele model, the major alleles of rs16891982 (G vs. C, combined OR [cOR]=2.74, 95% CI [2.20, 3.40]), rs885479 (G vs. A, cOR=1.46, 95% CI [1.06, 2.01]), rs1544410 (G vs. A, cOR=1.19, 95% CI [1.06, 1.34]), rs731236 (T vs. C, cOR=1.11, 95% CI [1.00, 1.23]), and the minor alleles of rs25487 (G vs. A, cOR=0.92, 95% CI [0.85, 0.99]), rs4911414 (G vs. T, cOR=0.85, 95% CI [0.75, 0.96]), rs1695 (W vs. M, cOR=0.79, 95% CI [0.65, 0.95]), and rs2228570 (wild-type allele vs. mutant allele, cOR=0.79, 95% CI [0.71, 0.88]) were related significantly to SC in at least two of the studies. The pooled P value for all SNPs was less than 0.05.

3.3.5 The dominant model (AA+AB vs. BB)

Table 3 of online supplementary file summarized the 52 SNPs' cOR for SC according to the dominant model. The results show that those who were homozygous and heterozygous for the major alleles: rs16891982 (GG+GC vs. CC, cOR=3.72, 95% CI [1.66, 8.35]), rs494379 (TT+TC vs. CC, cOR=2.62, 95% CI [1.96, 3.49]), rs514921 (AA+AG vs. GG, cOR=2.14, 95% CI [1.67, 2.75]), rs1144393 (AA+AG vs. GG, cOR=1.48, 95% CI [1.19, 1.84]), rs11615 (AA+AG vs. GG, cOR=1.41, 95% CI [1.02,

1.95]), and rs498186 (TT+TG vs. GG, cOR=1.35, 95% CI [1.10, 1.65]) had a higher risk for developing SC, than those homozygous for the minor alleles. In contrast, individuals homozygous for the minor alleles rs25487 (GG+GA vs. AA, cOR=0.85, 95% CI [0.72, 1.00]) and rs1805007 (CC+CT vs. TT, cOR=0.42, 95% CI [0.19, 0.91]) were significantly associated with increased susceptibility to SC.

3.3.6 Subgroup analysis

Covariate regression analysis was performed for each of the three genotypes. The results showed that cancer type was not the source of heterogeneity in the studied models (Table 3).

Table 3 Subgroup analyses according to the cancer type

	Study number	Sensitivity [95%CI]	p	Specificity [95%CI]	p	LRT Chi^2	p
Allele model	121	0.85 [0.78, 0.91]	0.15	0.17 [0.11, 0.25]	0.28	3.31	0.19
Dominant model	76	0.95 [0.90, 0.97]	0.44	0.19 [0.11, 0.31]	0.28	3.16	0.21
Recessive model	132	0.77 [0.65, 0.86]	0.05	0.35 [0.25, 0.47]	0.29	5.88	0.05

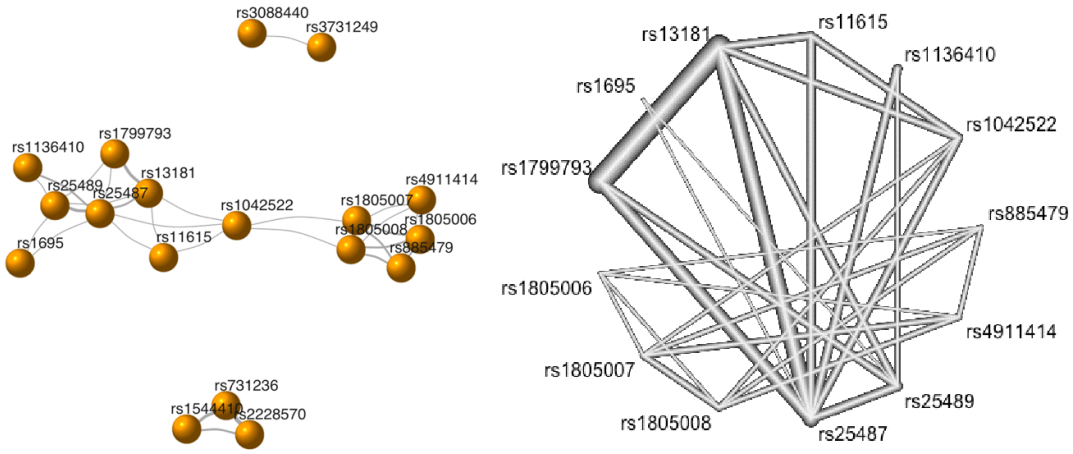
LRTChi2, Likelihood ratio test in joint model.

3.3.7 2.3.7 Network evidence

3.3.7.1 The allele model

The network plot depicts the rough comparison of each pair of SNPs (Figure 10). A node indicates an SNP, and its size represents the number of studies. The connections between the nodes mean a pair of comparisons and their thickness represents the number of direct comparisons. As is evident from Figure 10a, there were three

subgroups without any connections. Also, to avoid redundancy, the network of SNPs from one study was deleted in our study. Thus, the NMA of the allele model was divided into two groups: subgroup one (including rs1544410, rs2228570, and rs731236) and subgroup two (including rs1042522, rs1136410, rs11615, rs13181, rs1695, rs1799793, rs1805006, rs1805007, rs1805008, rs25487, rs25489, rs4911414, and rs885479) (Figure 10b).



a. Network plot of SNPs in all subgroup b. Network plot of SNPs in subgroup 2

Figure 8 The network evidence plot of single nucleotide polymorphisms (SNPs) in the allele model (A vs. B).

The SNPs rs731236 vs. rs2228570 had the strongest negative correlation with SC risk in subgroup one (standardized mean differences (SMD) of OR=-0.08, 95% CI [-0.18, 0.02]) (Table 4). However, the P values of the correlations between the SNPs in subgroup one were above 0.05.

Table 4 The direct and indirect evidence of each compairson in the subgroup one

Comparison	Direct	Indirect	Diff	Z	p-value
rs1544410 vs rs2228570	0.05	0.00	0.05	0.26	0.79
rs1544410 vs rs731236	-0.03	-0.04	0.01	0.04	0.97

rs2228570 vs rs731236	-0.08	-0.25	0.17	0.41	0.68

Similarly, as shown in Figure 6, Table 4 of online supplementary file, the comparison with the highest direct pooled effect size in subgroup two was rs4911414 vs rs1805006 (SMD of OR=-2.94, 95%CI [-2.48, -3.40]), followed by comparison rs13181 vs. rs25489 (SMD of OR=-2.35, 95% CI [-2.54, -2.16]).

Additionally, in subgroup two, the direct and indirect evidence showed negative correlations in the comparisons of rs1042522 vs. rs25487, rs1136410 vs. rs25489, rs11615 vs. rs13181, rs11615 vs. rs25487, rs13181 vs. rs1799793, rs13181 vs. rs25487, rs1805007 vs. rs1805006, and rs1805007 vs. rs885479 (Table 4 of online supplementary file). However, since the indirect evidence proportion of each comparison (i.e., the mean path length of each estimated comparison) was less than 2 (194) , each of the above mentioned comparisons followed the direction of direct evidence (Figure 11).

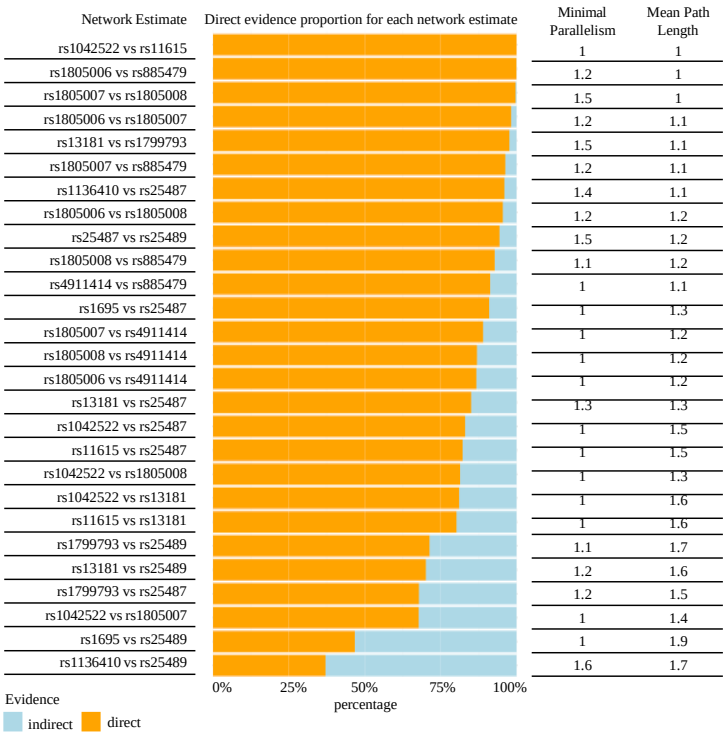


Figure 9 The percentage of direct and indirect evidence in the subgroup two of the allele model. The comparisons only with indirect evidence were hidden due to the

limited space.

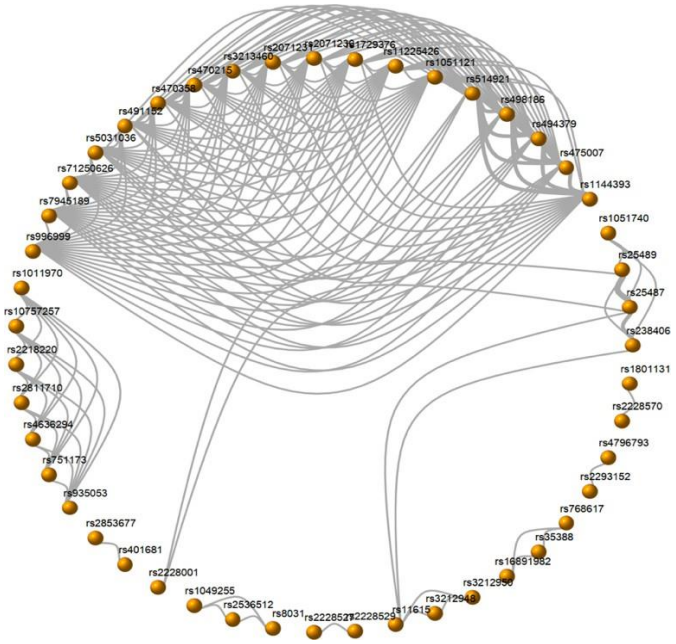
To select the SNPs with the highest chance of a significant association with skin cancer, the P scores were ranked, as shown in Table 5. The SNP rs2228570 (P-score=0.85) ranked first in subgroup one in the allele model and SNP rs13181 had the highest P-score in subgroup two (P-score=0.94).

Table 5 The rank of P-score of the SNPs in each subgroup in the Allele model

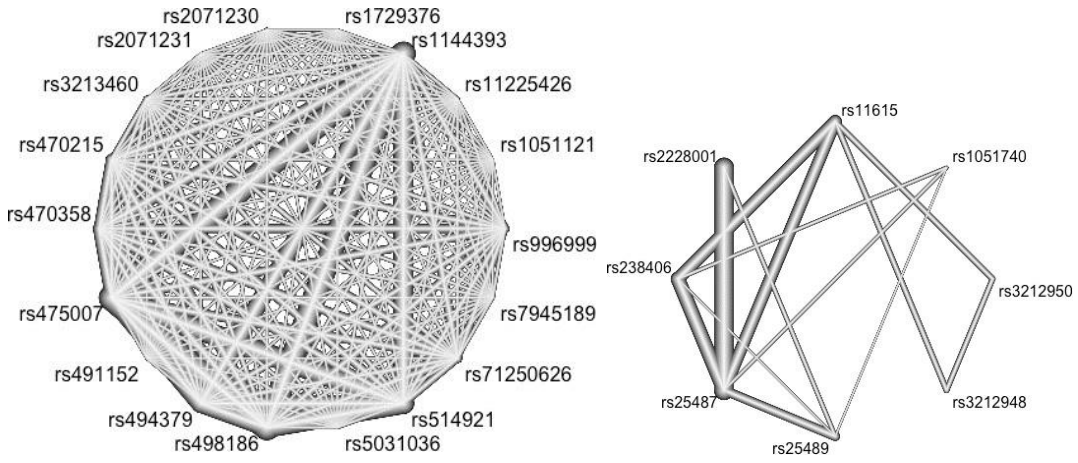
Rank	Subgroup 1	P-score	Subgroup 2	P-score
1	rs2228570	0.85	rs13181	0.94
2	rs1544410	0.47	rs1799793	0.90
3	rs731236	0.18	rs25487	0.88
4			rs11615	0.77
5			rs1042522	0.64
6			rs1695	0.57
7			rs4911414	0.54
8			rs1136410	0.41
9			rs1805007	0.33
10			rs1805008	0.24
11			rs25489	0.19
12			rs885479	0.08
13			rs1805006	0.00

3.3.7.2 The dominant model

In Figure 12a, only two subgroups met the requirements for the NMA. Subgroup one included rs1051121, rs11225426, rs1144393, rs1729376, rs2071230, rs2071231, rs3213460, rs470215, rs470358, rs475007, rs491152, rs494379, rs498186, rs5031036, rs514921, rs71250626, rs7945189, and rs996999 (Figure 12b), while subgroup two included rs1051740, rs11615, rs2228001, rs238406, rs25487, rs25489, rs3212948, and rs3212950 (Figure 7c).



a. Network plot of SNPs in all subgroups



b. Network plot of SNPs in subgroup 1 c. Network plot of SNPs in subgroup 2

Figure 10 The network evidence plot of single nucleotide polymorphisms (SNPs) in the dominant model (AA+AB vs. BB). a. network map with 47 SNPs; b. Supgroup one network map with 18 SNPs; c. Supgroup two network map with 8 SNPs.

There was no inconsistency between the direct and indirect evidence in group one. The strongest positive correlations in this subgroup, were the comparison of rs475007 vs. rs1729376 and the comparison of rs475007 vs. rs2071231 (both SMD of network

OR=4.23, 95% CI [2.19, 6.25]). These were followed by the rs475007 vs rs491152 comparison, which SNPs were negatively correlated with SC risk (SMD of network OR=-4.21, 95% CI [−6.24, −2.18]). The comparison of rs494379 and rs514921 showed the strongest indirect correlation (SMD of indirect OR=11.52, 95% CI [-9.40, 32.44]) (Table 5 of online supplementary file).

As shown in Figure 13, the direction of direct evidence and indirect evidence were different in the comparisions of rs1144393 vs rs1051121, rs11225426 vs rs1144393, rs1144393 vs rs1729376, rs1144393 vs rs2071230, rs1144393 vs rs2071231, rs1144393 vs rs3213460, rs1144393 vs rs470215, rs1144393 vs rs470358, rs1144393 vs rs491152, rs1144393 vs rs5031036, rs1144393 vs rs71250626, rs1144393 vs rs7945189, rs1144393 vs rs996999, rs470215 vs rs514921, rs470358 vs rs498186, rs475007 vs rs514921, rs498186 vs rs514921, rs514921 vs rs71250626. However, because their indirect evidence proportion of each comparison was less, followed the direction of direct evidence (Figure 14).

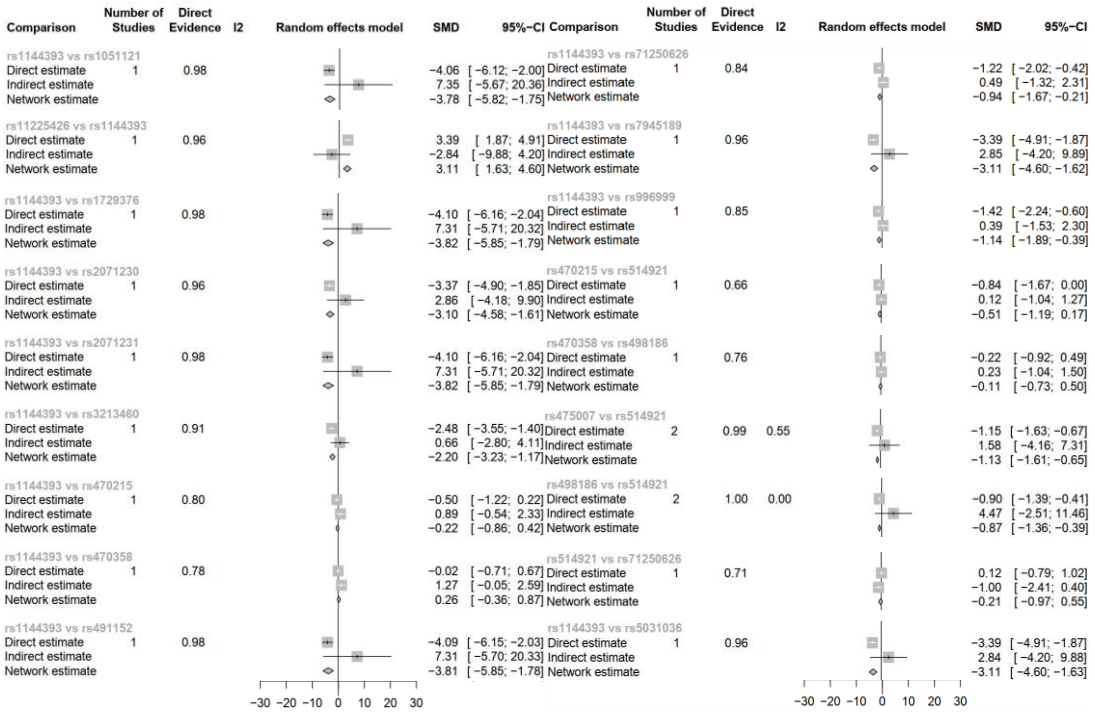


Figure 11 The direct and indirect evidence forest plot of subgroup one in the dominant model with different direction.

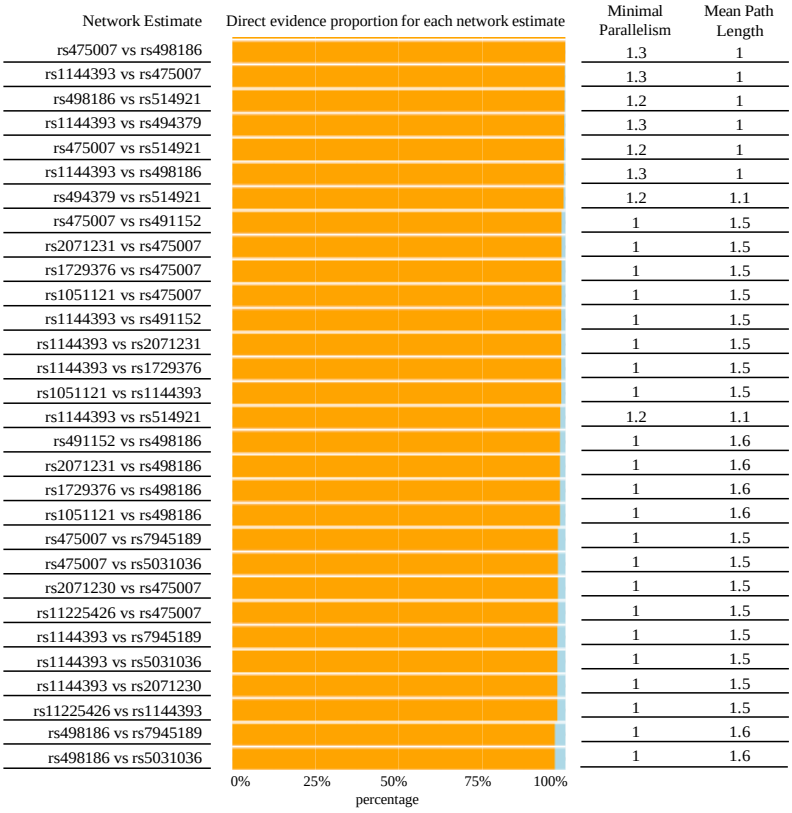


Figure 12 The percentage of direct and indirect evidence in the subgroup one of the dominant model. The comparisions only with indirect evidence were hidden due to the limited space.

In the subgroup two, direct and indirect evidence inconsistencies were found in the comparison of rs2228001 vs. rs25487 and the comparison of rs2228001 vs. rs25489

(Table 5 of online supplementary file). While the percentage of direct evidence of both these two comparisons were large than the indirect evidence (Figure 15). Hence, as shown in Figure 11, both rs25487 and rs25489 negatively correlated with rs2228001 after the network analysis. Figure 16 also showed the rs238406 vs. rs25489 comparison had the strongest relationship (SMD of network OR=-2.17, 95% CI [−2.72, −1.61]).

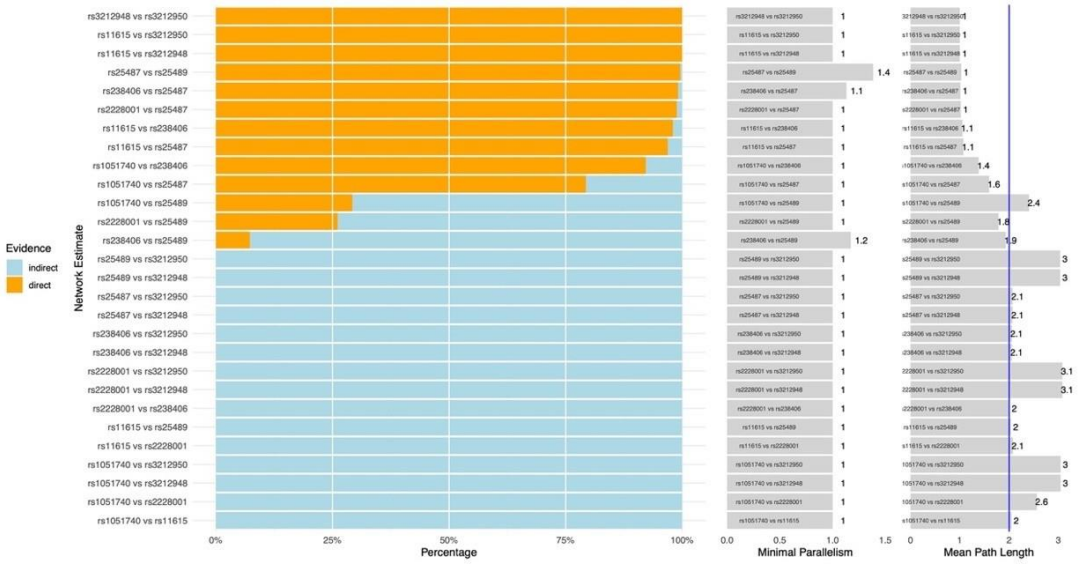


Figure 13 The percentage of direct and indirect evidence in the subgroup two of the dominant model.

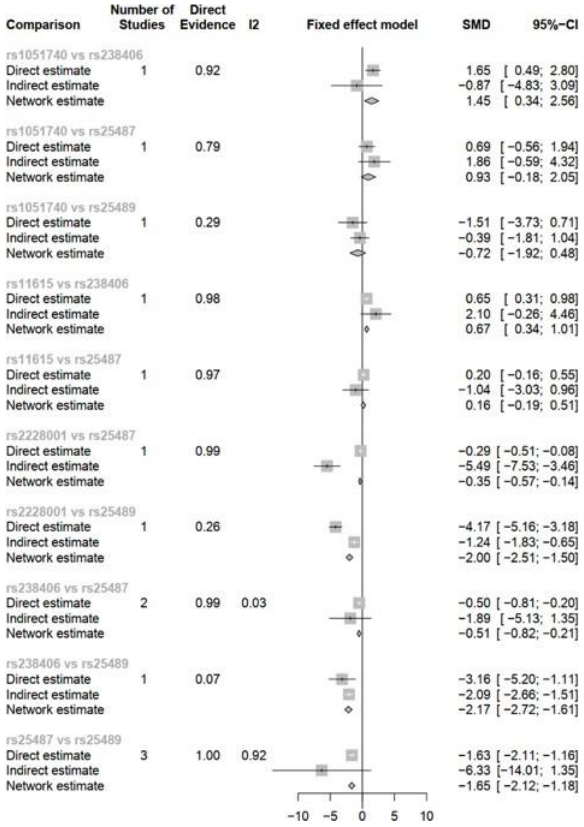


Figure 14 The direct and indirect evidence forest plot of subgroup two in the dominant model.

As shown in Table 6, rs475007 has the highest P-score (0.97) in subgroup one and rs238406 has the highest P-score (0.97) in subgroup two. Therefore, the top five SNPs most likely associated with skin cancer in descending order, in subgroup one, are: rs475007, rs470358, rs498186, rs1144393, rs470215, and in subgroup twoare : rs238406, rs2228001, rs25487, rs11615, rs3212950.

Table 6 The rank of P-score of the SNPs in each subgroup

Rank	Subgroup 1	P-score	Subgroup 2	P-score
1	rs475007	0.97	rs238406	0.97
2	rs470358	0.92	rs2228001	0.87
3	rs498186	0.89	rs25487	0.62
4	rs1144393	0.84	rs11615	0.50

5	rs470215	0.79	rs3212950	0.41
6	rs514921	0.68	rs3212948	0.41
7	rs71250626	0.62	rs1051740	0.21
8	rs494379	0.59	rs25489	0.02
9	rs996999	0.58		
10	rs3213460	0.42		
11	rs2071230	0.27		
12	rs7945189	0.27		
13	rs11225426	0.26		
14	rs5031036	0.26		
15	rs1051121	0.17		
16	rs491152	0.16		
17	rs1729376	0.16		
18	rs2071231	0.16		

3.4 Discussion

Based on direct comparisons of pairwise meta-analysis and added indirect comparisons, our study employed the network meta-analysis to compare the associations between single-nucleotide polymorphisms and skin cancer using the allele model and the dominant model. Our network meta-analysis identified two subgroups in each genetic model, respectively. We ranked SNPs based on their P-score to select the most appropriate SNPs. Our results showed, that the minor alleles (T) of rs2228570 (FokI) and (C) of rs13181(ERCC2) were the highest-ranking SNPs, in both subgroups one and two, in the allele model. On the other hand, using the dominant model, the wildtype and heterozygous alleles (AA+AT) of rs475007 in subgroup one and the mutated homozygous allele (AA) of rs238406 in subgroup two were most likely to be associated with skin cancer.

The single-nucleotide polymorphism rs2228570 (FokI) is located in the vitamin D receptor (VDR) gene. It is one of the common human VDR SNPs along with

rs1544410(BsmI), rs7975232 (ApaI) and rs731236 (TaqI). Vitamin D is metabolized to vitamin D: 1,25(OH)2D3.1 in response to ultraviolet B (UVB) radiation. This metabolite is the ligand of the VDR, which in turn initiates a series of biological responses in bone metabolism, immunity, cell proliferation, and differentiation by binding to vitamin D response elements in the DNA (195). Hence, rs2228570 has not only been associated with various skin diseases, such as chronic spontaneous urticaria (CSU) (196), atopic dermatitis (AD) (197), and leprosy (198), but has also been linked to an increased incidence risk and worse prognosis of different cancers, such as breast cancer (42), ovarian cancer (43), gastric cancer (44), hepatocellular carcinoma (45), papillary thyroid cancer (46), pancreatic cancer (47) and melanoma. Our results are consistent with previous studies using assay methods (199). For instance, the study results of Zeljic et al, who used the assay method showed that the mutated genotype of rs2228570 was related to increased melanoma risk compared to the wildtype genotype in the Caucasian population (199). However, no association was observed between rs2228570 and melanoma in this investigation using the biosystem assay method (200).

SNPs rs13181 and rs238406 ranked first and second in subgroups two in both the allele and the dominant models. Both SNP alleles are located in the ERCC2 (formerly called XPD) gene. The ERCC2 polymorphisms have an ATP-dependent DNA helicase activity, which may impact DNA repair functions. Deficiency of ERCC2 has been reported to lead to xeroderma pigmentosum (XP), trichothiodystrophy (TTD), and Cockayne's syndrome (CS) (201). This observation may explain why rs13181 and rs238406 were found to be linked to cancers, such as lung cancer (48), cervical cancer (49), breast cancer, squamous cell carcinomas of the head and neck (50), and bladder cancer (51). In line with these findings, our results showed that the minor allele (C) of rs13181 and the mutated homozygous allele (AA) of rs238406 were significantly associated with SC risk. The study by Kertatbs et al. reported high frequency of the wild type allele of rs13181 in advanced melanoma (202). However, an investigation using the microarray chip method including 1,391 NMSC cases and 2,586 cancer-free controls did not find significantly increased risks of NMSC for wildtype rs13181 (203). Furthermore, a

meta-analysis found that the mutated homozygous allele (AA) of rs238406 was positively associated with the increased risk of cancer of the nervous system, the digestivetract , the genito-urinary system, and the respiratory system, but without basal cell cancer (204).

The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are involved in cell mobility, proliferation, differentiation, and apoptosis by degrading extracellular proteins (205). MMP1, a secreted enzyme that cleaves fibrillar collagen, has been linked to cancer, by promoting cancer cell proliferation, tumor angiogenesis and vasculogenesis (206). In the dominant model of our research, all the SNPs from subgroup one were located in the MMP1 gene, and the SNP most likely to be associated with SC was rs475007. Furthermore, homozygosity for the minor allele of rs475007 was found to decrease the risk of skin cancer. Similar results were found in Hongliang Liu's study, which reported that patients homozygous or heterozygous for the major allele of rs475007 were more likely to have larger skin tumors (207).

3.5 Limitation

Due to technical differences and differences in sensitivity, our analysis only included studies that used the PCR genotypic detection method and excluded the microarray detection or genome-wide association studies (GWAS). However, GWAS allow for much larger sample sizes than PCR studies. Additionally, due to the limitations of the RStudio and StataSE softwares and the complexity of multi-arm studies, SNPs only reported in one single article were not included in the final network meta-analysis.

3.6 Future prospective

Our article indicated that people with mutations in the genes FokI (rs2228570), ERCC2 (rs13181), MMP1(rs475007) and ERCC2 (rs238406) were more likely to have skin cancer. Dysplastic nevi (also called atypical moles) are precursors and risk factors for malignant melanoma (208). However, it is difficult to distinguish them from

melanomas because of overlapping features and lack of predictive markers(209). Thus, our results may provide a possibility for the early detection of asymptomatic skin cancer if routine genetic screening is implemented in the general population in the future. Additionally, the results of our study may also provide valuable information for decision-making when determining the best mode of therapy of SC in a patient. For instance, since FokI is a vitamin D receptor gene and vitamin D is considered to be a protective factor in certain cancers, such as skin cancer (210,211), supplementation with Vitamin D may be used as adjuvant therapy in cancer patients. Therefore, identification of SC patients with FokI gene (rs2228570) mutations is important, since these patients would not benefit from adjuvant Vitamin D therapy.

In addition, we obtained direct and indirect evidence between the SNP pairs through network analysis, which proposed the possibility of hitherto unexplored relationships between certain gene mutations. For example, ERCC2 gene mutations have been shown to indirectly increase the risk of SC (212,213), and the melanocortin receptor 1 (MC1R), which encodes melanocyte-stimulating hormone (MSH) receptors, has also been shown to be risk factor for skin cancer (214). However, surprisingly, the indirect evidence of our network meta-analysis showed that ERCC2 (rs13181) was negatively related to MC1R (1805006, 1805007, 1805008, and rs885479). Therefore, the relationship between ERCC2 and MC1R, necessitates further research to determine their role in SC development.

Finally -as added scientific value - , we applied an innovative research design by performing a network analysis of case-control studies, thus providing a fresh perspective on the NMA method. Our analysis implies, that all studies involving genetically-related diseases, whether they are cohort or case-control studies, can be used to build a network in the meta-analysis, which may then provide as valuable information for the diseases' early detection, diagnosis, staging, treatment and prognosis.

4 New results

1) The expression of LINC-PINT and lincRNA-P21 upregulation with the concentration of H_2 gas.

2) LINC-PINT expression decreased in a relatively long H_2 usage time

3) The expression of lincRNA-P21 declined with the H_2 concentration.

4) The minor alleles of rs2228570 (FokI) and rs13181(ERCC2) were associated with skin cancer.

5) Wildtype and heterozygous genotypes of rs475007 (MMP1) and the mutated homozygous genotype of rs238406 (ERCC2) were most likely to be associated with skin cancer.

5 Conclusions

The expressions of LINC-PINT and lincRNA-P21 were upregulated after H_2 gas treatment. And the SNP rs2228570 (FokI), rs13181(ERCC2), rs475007 (MMP1) and rs238406 (ERCC2) can be employed as the early biomarkers for skin cancer.

6 Acknowledgements

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7 List of Publications

7.1 Thesis based articles

- The relationship between single nucleotide polymorphisms and skin cancer susceptibility: A Systematic Review and Network Meta-Analysis (IF=6.24)

Lu Zhang, Eva Pozsgai, Yongan Song, John Macharia, Huda Alfatafta, Jia Zheng, Zhaoyi Li, Hongbo Liu, István Kiss

Front Oncol (2023) 13:1094309. doi: 10.3389/fonc.2023.1094309

- The therapeutic effect of hydrogen gas on lung cancer - LINC-PINT and lincRNA-P21 as biomarkers

Lu Zhang, Timea Varjas, Eva Pozsgai, István Szabó, Ágnes Szenczi, Huda Alfatafta, Yongan Song, John Macharia, Hongbo Liu, István Kiss (IF=1.4)

Genetic Testing and Molecular Biomarkers. Submitting for publication

7.2 Other publications

- Lower handgrip strength levels probably precede triglyceride glucose index and associated with diabetes in men not in women

Jia Zheng, Lu Zhang, Min Jiang

Journal of Diabetes Investigation, vol. 13, no. 1, pp. 148–155, 2022, doi: 10.1111/jdi.13626.

- Are chemical compounds in medical mushrooms potent against colorectal cancer carcinogenesis and antimicrobial growth?

John M. Macharia, Lu Zhang, Ruth W. Mwangi, Nora Rozmann, Zsolt Kaposztas, Tímea Varjas, Miklós Sugár, Huda Alfatafta, Márton Pintér, Raposa L. Bence

Cancer Cell Int, vol. 22, no. 1, p. 379, Dec. 2022, doi: 10.1186/s12935-022-02798-2.

- Microarray data analysis to identify miRNA biomarkers and construct the lncRNA-miRNA-mRNA network in lung adenocarcinoma

Yongan Song, Leonardo Kelava, Lu Zhang, István Kiss

Medicine, vol. 101, no. 36, p. e30393, Sep. 2022, doi: 10.1097/MD.0000000000030393.

- Effect of the knee replacement surgery on activity level based on ActivPAL: a systematic review and meta-analysis study

Alfatafta Huda, Alfatafta Mahmoud, Onchonga David, Hammoud Sahar, Khatatbeh Haitham, Zhang Lu, Boncz Imre, Lohner Szimonetta, Molics Bálint

Musculoskeletal Disorders, vol. 23, no. 1, p. 576, Jun. 2022, doi: 10.1186/s12891-022-05531-2.

- Effect of using knee valgus brace on pain and activity level over different time intervals among patients with medial knee OA: systematic review

Huda Alfatafta, David Onchonga, Mahmoud Alfatafta, Lu Zhang, Imre Boncz, Szimonetta Lohner, Bálint Molics

BMC Musculoskeletal Disorders, vol. 23, no. 1, p. 576, Jun. 2022, doi: 10.1186/s12891-022-05531-2.

- Focusing on the premature death of redeployed miners in China: an analysis of cause-of-death information from non-communicable diseases

Wei Xian, Bing Han, Leizhen Xia, Yining Ma, Haodi Xu, Lu Zhang, Li Li, Hongbo Liu

Global Health, vol. 15, p. 7, Jan. 2019, doi: 10.1186/s12992-019-0450-5.

- Effect of socio-demographic characteristics on social security in northeast China

Wei Xian, Cheng Jin, Bing Han, Xueying Xu, Lu Zhang, Hongbo Liu

Health Soc Care Community, Aug. 2020, doi: 10.1111/hsc.13127.

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9 Appendices

9.1 Appendice 1 Submission of the doctoral dissertation and declaration of the originality of the dissertation

The undersigned,
Name: Lu Zhang
Maiden name: Lu Zhang
Mother’s maiden name: Genlan Jing
Place and time of birth: HEBEI, China. 21 Auguest 1992

on this day submitted my doctoral dissertation entitled: Research on the application of molecular epidemiology in the prediction and prognosis of cancer

to the
PR-1, frontiers of health sciences Programme
of the Doctoral School of Health Sciences, Faculty of Health Sciences, University of

Pécs.

Names of the supervisor(s): Prof. Dr. Kiss István and Prof. Dr. Hongbo Liu

At the same time, I declare that

- I have not submitted my doctoral dissertation to any other Doctoral School (neither in this country nor abroad),
- my application for degree earning has not been rejected in the past two years,
- in the past two years I have not had unsuccessful doctoral procedures,
- my doctoral degree has not been withdrawn in the past five years,
- my dissertation is independent work, I have not presented others' intellectual work as mine,

the references are definite and full, on preparation of the dissertation I have not used false or falsified data.

Dated: 3 December 23

LU ZHANG

signed by Candidate

Supervisor

Co-supervisor