

Research status and prospects of biomarkers for nasopharyngeal carcinoma in the era of high-throughput omics (Review)

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Abstract. As a malignant tumor type, nasopharyngeal carcinoma (NPC) is characterized by distinct geographical, ethnic and genetic differences; presenting a major threat to human health in many countries, especially in Southern China. At present, no accurate and effective methods are available for the early diagnosis, efficacious evaluation or prognosis prediction for NPC. As such, a large number of patients have locoregionally advanced NPC at the time of initial diagnosis. Many patients show toxic reactions to overtreatment and have risks of cancer recurrence and distant metastasis owing to insufficient treatment. To solve these clinical problems, high-throughput '-omics' technologies are being used to screen and identify specific molecular biomarkers for NPC. Because of the lack of comprehensive descriptions regarding NPC biomarkers, the present study summarized the research progress that has been made in recent years to discover NPC biomarkers, highlighting the existing problems that require exploration. In view of the lack of authoritative reports at present, study design factors that affect the screening of biomarkers are also discussed here and prospects for future research are proposed to provide references for follow-up studies of NPC biomarkers.

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1. Introduction

As a type of malignant head and neck tumor, nasopharyngeal carcinoma (NPC) is characterized by early extensive local infiltration, high hematogenous dissemination, early lymphatic spread and a high mortality rate (1). According to the International Agency for Research on Cancer (IARC), there were an estimated 129,079 new cases of NPC and 72,987 NPC-related deaths in 2018 worldwide (2). While this is only a small proportion of all cancers (the number of NPC cases in 2018 accounted for 0.7% of all confirmed cancers), in some high-risk countries or regions, many patients present with NPC as well as drug toxicity due to anti-NPC treatment. China has the highest incidence rate of NPC worldwide. In 2018, there were 60,558 new NPC cases (47.7% of total global cases) and 31,413 NPC-related deaths in China (1,2). Within China, the incidence rate of NPC is highest in the Guangdong Province; hence, NPC is also commonly referred to as 'Cantonese cancer' (3). NPC has a high incidence rate in other provinces of China, such as Guangxi, Hunan and Fujian, as well as in other countries, such as Indonesia, Vietnam, India, Morocco, Algeria, Tunisia and Ghana (4). Therefore, the prevention and treatment of NPC is an important goal for medical researchers.

To date, the pathogeny behind NPC remains unclear. Existing evidence shows that heredity, race, environment, diet, habits and the Epstein-Barr virus (EBV) are closely related to the pathogenesis behind NPC (1). However, the study showed that NPC development involves a complex interaction of multiple factors, as demonstrated by some gene polymorphisms with different genetic characteristics that are carried by ethnic groups in high-risk areas being associated with different degrees of NPC risk as well as the processing and presentation of EBV antigens. Extracts of Cantonese salted fish from China, as well as herbal medicines used by Chinese and Naga people, have been shown to promote the activation and

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proliferation of EBV, leading to the occurrence of NPC (5). In addition, EBV inducers are detected in soil extracts from NPC-endemic areas in southern China as well as in some vegetables grown in these soils (6). These factors attribute for the increased NPC incidence rate observed in specific regions or races. However, how the genetic factors of different races affect tumor development still requires more systematic and comprehensive research.

Effective early diagnosis and treatment are key to preventing the progression, recurrence and metastasis of NPC. Based on the guidelines published by the World Health Organization (WHO), pathological evidence found by biopsies is the gold standard for NPC diagnosis (7-9). However, because of poor compliance of patients with this invasive procedure, the fact that the symptoms of NPC are not conclusive at early stages and that NPC is not conducive to imaging screening, more than 70% of patients have locoregionally advanced (LA)-NPC at the time of initial diagnosis (10). Because the location of the NPC is close to the base of the skull and its shape is irregular, it is difficult to distinguish the tumors from nasopharyngeal lymphoma in the early stage using MRI or computed tomography scanning (11). Furthermore, imaging-dependent tumor staging is initially established based on patient survival statistics and is often affected by the subjective judgement of the clinician; therefore, a reliable laboratory method is needed to assist in accurate NPC diagnosis (4,12).

The treatment decision for NPC at different stages is made based on the tumor-node-metastasis (TNM) staging system developed by the Union for International Cancer Control/American Joint Committee on Cancer and the National Comprehensive Cancer Network (NCCN). Radiotherapy (RT) alone is the standard treatment for early NPC, while for patients with stages III-IVa, induction chemotherapy (IC) followed by concurrent chemoradiotherapy (CCRT) or CCRT followed by adjuvant chemotherapy (AC) are the preferred category 2A treatment regimen (4,12). A number of clinical trials have investigated the effectiveness of these treatment methods (10,13-15). Although the 5-year overall survival (OS), progression-free survival (PFS), distant metastasis-free survival (DMFS) and locoregional relapse-free survival (LRFS) are improved to various degrees by the application of RT or the one of the various chemotherapy (CT) regimens, there are still many controversies in the clinical application of these treatment methods because of the lack of accurate efficacy evaluation indicators. For example: i) Some patients still have tumor recurrence or distant metastasis after receiving radical RT or IC+CCRT/CCRT+AC, indicating that a more effective treatment should be developed (16-19); ii) IC+CCRT improves the OS and DMFS of patients with LA-NPC to a limited degree, but most patients do not benefit from it, suggesting overtreatment (13-15); iii) patients have poor tolerance to toxic side effects of the treatment, which could lead to delays or interruptions in treatment, thus increasing the risk of tumor progression or drug resistance (1,20-22); and iv) the clinical use of Response Evaluation Criteria in Solid Tumors (RECIST) can only provide a reference for the NPC treatment effect evaluation through generalized remission or progression, and cannot provide quantifiable prediction indexes for NPC recurrence and metastasis. Moreover, the prognosis model based on the TNM staging system cannot provide an objective

assessment for the risk of disease progression for patients with NPC (23,24). These problems indicate issues regarding NPC diagnosis, treatment and prognosis, and suggest the need for individualized treatment. Therefore, researchers are focusing on the identification of molecular biomarkers to establish new non-invasive early diagnostic methods, ideal treatment regimens, efficacy evaluation standards and prognostic indicators for NPC (Fig. 1).

Biomarkers can be divided into two categories according to their functions: Those used to discover the molecular mechanisms of action of the disease and drug targets, and those used for the prediction, early diagnosis, efficacy evaluation and prognosis. Several review articles have summarized both types of NPC biomarkers (7-9,25). With the development of high-throughput omics technologies in recent years, many novel biomarkers have been discovered. In the present review, studies on bodily fluid samples, such as serum, plasma and saliva of patients with NPC in the past 5 years were summarized, and the research status of biomarkers for the early diagnosis, treatment (such as RT and CT) and prognosis (such as metastasis and recurrence) of NPC were reviewed at the aspects of genomics, transcriptomics, proteomics and metabolomics. The intersection points (such as TNM staging, therapeutic method, sample type and high-throughput technology) in the study design of the existing research and the development trends of NPC biomarkers in the future were also discussed.

2. NPC biomarkers discovered through genomics

Genomics aims to collectively characterize and quantify all genes in an organism to study the influence of their interactions (26). It uses high-throughput DNA sequencing, bioinformatics, genetic analysis and functional identification to analyze the structure and function of the whole genome, which forms the omics framework of systems biology with transcriptomics, proteomics and metabolomics (27). In the study of biomarkers, gene mutations are often difficult to match with corresponding clinical phenotypes of the disease. Therefore, researchers must carry out repeated validation studies in large cohorts or conduct supplementary studies with other omics technologies (28).

Based on the fact that genetics, environment, diet and EBV are generally considered as risk factors of cancer, exploring new pathogenic genes and mechanisms to assess the risk of NPC in high-risk population has become the goal of genomics research (29,30). With the wide application of high-throughput technologies, such as whole-exome sequencing, whole-genome sequencing (WGS) and genome-wide association studies (GWAS), many single nucleotide polymorphisms (SNPs) associated with NPC have been identified and comprehensively described (1,7,30-32). However, although the interactions between multiple genes make the complex mechanism of action behind NPC more difficult to understand, simple research design and mature detection technology still push the research forward (33). In recent years, researchers have identified the association between NPC susceptibility and the SNPs of genes encoding for major histocompatibility complex II (34), monocyte chemoattractant protein-1 promoter (35), xeroderma pigmentosum group D, human 8-oxoguanine

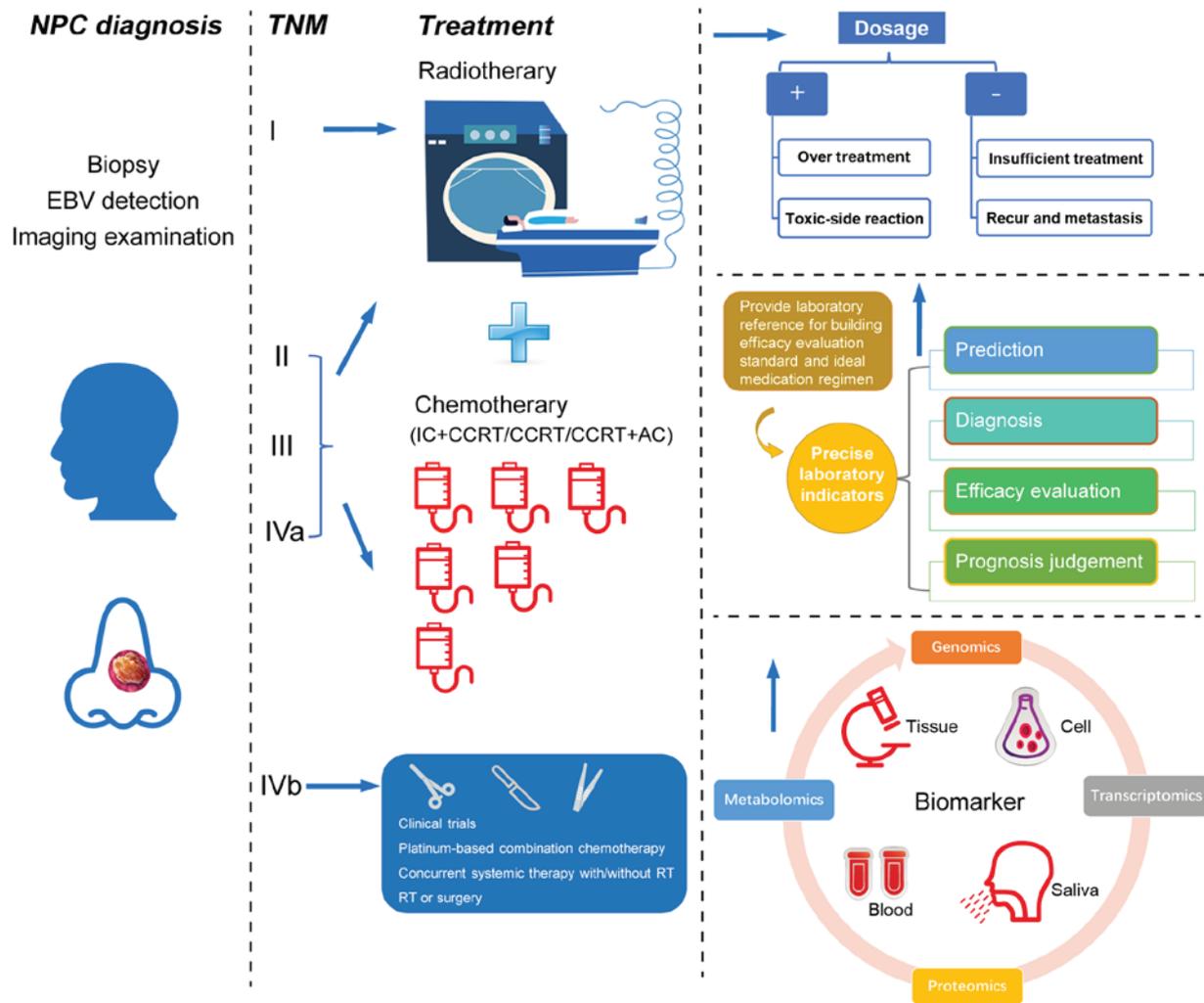


Figure 1. Role of biomarkers in NPC diagnosis, treatment, and prognosis. Biomarkers can effectively predict the risk of NPC incidence and reduce the number of patients with a locoregionally advanced stage through early diagnosis. For patients at different stages, biomarkers can effectively assist physicians to carry out clinical individualized treatment. This includes creating ideal regimens to improve the treatment effect and providing targets for the development of novel medication which avoid the toxic reactions caused by treatment. Effective biomarkers may also be used to accurately evaluate the prognosis of patients, as well as reduce the recurrence and distant metastasis of tumors. AC, adjuvant chemotherapy; CCRT, concurrent chemoradiotherapy; EBV, Epstein-Barr virus; IC, induction chemotherapy; NPC, nasopharyngeal carcinoma; TNM, tumor-node-metastasis.

DNA glycosylase 1 (*hOGG1*), integrin (ITG) α 2 (36) and cyclophilin A26 (37) in patient cohorts from various regions, and confirmed the effect of loss-of-function mutations such as negative regulator NF- κ B inhibitor α and cylindromatosis on NF- κ B activity and NPC cell growth, respectively (38,39). To date, no susceptibility genes have been approved for the early screening of NPC. The heterogeneity in the sample cohorts of different regions, races and pathological backgrounds is often highlighted as the main reason for the variation between the research results (7). Nevertheless, to the best of our knowledge, most studies did not verify the potential pathogenic genes. Some scholars think that because SNPs identified by GWAS are mostly minor alleles; therefore the main difficulty in further research, the large samples and failure to verify the downstream function of non-coding regions are the main reasons for the difficulty further research (33). In addition, the integration of multi-disciplinary research may provide a new method to interpret this complex problem, such as the joint effect of some specific regional environmental factors and traditional unhealthy diets on the incidence of NPC (40-42).

However, finding the interdisciplinary intersection point seems to be beyond the research objective itself.

In a whole exon sequencing study of 251 individuals from 97 polygenic families in Taiwan, Yu *et al* (43) found that 12 gene variations related to magnesium transport (Nuclear-interacting partner of anaplastic lymphoma kinase-like domain containing 1), EBV cell entry (ITG- β 6), modulation of EBV infection (bcl-2-like protein 12; and neural precursor cell expressed, developmentally down-regulated 4-like), telomere biology (cleft lip and palate transmembrane 1 like; bromodomain containing 2; and heterogeneous nuclear ribonucleoprotein U), modulation of cAMP signaling (rap guanine nucleotide exchange factor 3), DNA repair (protein kinase, DNA-activated, catalytic subunit; and mutL homolog 1) and the Notch signaling pathway (notch receptor 1; and δ -like canonical notch ligand 3), play important roles in activating T cells to respond to EBV infection. Liu *et al* (44) verified these mutations in a later study and emphasized the important role of telomere length maintenance in NPC etiology. Two other studies from the USA and Tunisia showed the potential of

mixed-lineage leukemia 3; major histocompatibility complex, class I, A*26; major histocompatibility complex, class I, A*30; and major histocompatibility complex, class II, DR β 1*10 in screening high-risk NPC family members, respectively (45,46). The study pointed out that the relative potential risk of NPC among family members who have first-degree relatives diagnosed with NPC is increased eight-fold (47). For some high-risk countries or regions, it is still necessary to strengthen the mining of familial NPC susceptibility genes for the development of related gene detection and early diagnosis.

Patients with NPC can develop various degrees of oral mucositis, xerostomia, myelosuppression and other toxic reactions while receiving intensity-modulated RT (IMRT) (48). Furthermore, cancer recurrence and distant metastasis can occur in 30-40% of patients with NPC after treatment, indicating that the response of NPC to RT is subject to individual variation (49). Because tumor cells can repair radiation-induced damage in various ways, some genes related to radiosensitivity are often used to predict the therapeutic effect and prognosis of NPC (16,48-50). Ma *et al* (51) analyzed the genotypes of angiogenesis-related genes in 180 patients with NPC using Sequenom MassARRAY and found that EDN1-rs1800541, rs2071942 and rs5370 can be used as risk predictors of radiation-induced oral mucositis, xerostomia and myelosuppression, respectively. Le *et al* (52) screened SNPs of 24 patients with NPC by WGS and found that rs11081899-A, located in the 5'-untranslated region of the zinc finger protein 24 gene, is the genetic predisposing factor of radiation-induced oral mucositis. Yu *et al* (53) analyzed 9 potential functional SNPs in four genes in the Wnt/ β -catenin pathway and found that patients carrying catenin β -1 rs1880481, rs3864004; glycogen synthase kinase-3 β rs3755557; or adenomatous polyposis coli rs454886, may have a poor prognosis and can develop radiation-induced dermatitis and oral mucositis. Furthermore, several studies have discussed the potential of SNPs of different genes, such as X-ray repair cross-complementing 1 (*XRCC1*) rs25489, *XRCC1* Codon399, valosin-containing protein rs2074549 and calcitonin receptor rs2528521 in the base excision repair pathway and the endoplasmic reticulum stress pathway to predict the therapeutic effect and toxic reactions in NPC (54-57). However, it is worth noting that the cases involved in these studies included a number of patients with stages III-IVa NPC. Although these patients also needed standard RT, the additional effect of chemotherapeutic drugs in CCRT on blood composition is an important factor that cannot be ignored. Tan *et al* (58) found that the vascular endothelial growth factor-460C allele had a significant association with NPC invasiveness of grades 2-3 cervical lymph node metastasis and compared with CCRT, in NPC with-460 T/C polymorphisms, patients may not benefit from IC+CCRT in terms of the OS, LRFS, DMFS and PFS, which again emphasizes the contradicted value of using IC for clinical application.

In addition to the inherent susceptibility genes of humans, EBV, a category 1 carcinogenic virus identified by IARC, is detectable in 100% of patients with undifferentiated non-keratinizing NPC in endemic areas (especially in East and Southeast Asian countries, such as China). The mechanisms of EBV infection, carcinogenesis, modification of epigenetic profiles, immune escape and maintenance of tumor

cell survival are deeply understood and have been previously described (59). Therefore, based on this close relationship, EBV DNA or antibodies can be used as an early diagnostic tool to screen for patients with NPC. In recent years, the A157154C polymorphism of the A73 gene and the RPMS1 genotype of EBV have been identified for their susceptibility to NPC (60,61). In particular, the combination of EBV-viral capsid antigen (VCA) IgA and EBV-early antigen IgA showed better sensitivity and specificity than EBV-DNA in screening NPC high-risk family individuals (62). However, a large cohort study showed that the sensitivity and specificity of plasma EBV-DNA for the diagnosis of early asymptomatic NPC were 97.1 and 98.6%, respectively (63). The authors speculated that the difference in the results may be owing to variations of EBV DNA content in the serum or plasma, clinical stage and pathological classification of the patients (64). Furthermore, previous studies have found no significant difference in the exact load of viral nucleic acid between patients with NPC and non-cancerous controls, and an increase in EBV levels was noted in asymptomatic carriers and patients with mononucleosis and pharyngitis (65,66). Banko *et al* (67) found that P-Thr-sv-5 showed carcinoma-specific Epstein-Barr nuclear antigen 1 (*EBNA1*) variability in tissues and plasma of patients with undifferentiated NPC, monocytosis syndrome and renal transplantation by using nested PCR and considered that the identification of this subvariant should be used as a viral screening marker for the identification of NPC. However, further *in vitro* experiments are needed to confirm whether the additional amino acid substitution of this subvariant will affect the function of EBNA1 in regulating the replication and transcription of EBV genes and/or change the effect of EBNA1 on the transcription of other genes in host cells (68). In terms of efficacy evaluation and prognosis, Hui *et al* (69) detected SNPs of the excision repair 1 endonuclease non-catalytic subunit (*ERCC1*) gene in the plasma of patients with EBV-positive and EBV-negative NPC after RT in a clinical trial. The results showed that the ERCC1 C118T genotype was a powerful predictor for a good prognosis of patients with EBV-negative NPC, which could confirm the effect of RT and avoid serious toxic reactions caused by the additional adjuvant CT. Nevertheless, in view of some potential influencing factors mentioned in the aforementioned study, whether the objectivity of the results can be affected by different clinical stages, treatment methods and other confounding factors remains to be further explored.

3. NPC biomarkers discovered through transcriptomics

Transcriptomics studies gene transcription and its regulation at the RNA level (70). It explores variations in gene expression using sequencing technology and reveals the mechanism of specific regulatory genes in a pathological condition through cell phenotypes and functional research (71). In contrast to genomics, transcriptomics emphasizes the concept of time and space, which enables it not only to identify the phenotypic attribution of cells, but also to distinguish subtypes of diseases and to discriminate the various reactions caused by medications, as well as describe the survival rates of patients (72).

Previous studies have shown that micro RNAs (miRNAs/miRs) can play an important role in the occurrence,

invasion, metastasis and immune escape of NPC, and their stable expression in the peripheral circulation can not only be used as reliable markers for the early diagnosis of NPC but may also help in the effective prediction of the therapeutic effect and prognosis (73-75). Therefore, studies into miRNAs have gained a leading position in the field of transcriptomics in recent years (73). Recently, several miRNAs with good diagnostic ability have been found using PCR or microarray technology (76-83). Two types of EBV-encoded miRNAs, miR-BamHI A rightward transcripts (BART)7-3p and miR-BART13-3p, showed a remarkable potential for the early diagnosis of NPC (76). It is worth noting that as the first virus-encoding miRNAs, 44 mature miRNAs encoded by EBV have been confirmed to play an important regulatory role in the carcinogenesis and progression of NPC. Although the function and clinical value of these miRNAs has been explored comprehensively, some of the identified biomarkers still lack functional verification (84,85). In addition to the aforementioned problems, most of these studies lack functional analysis of the identified potential biomarkers. Because of the small molecular weight of miRNAs and their low content in the serum or plasma, it is difficult to fully reflect the expression abundance of these biomarkers. Therefore, the sensitivity and specificity of miRNAs, especially EBV miRNAs, were not satisfactory (78,80). Furthermore, although a previous study confirmed the mechanism of action of miRNAs in various stages of NPC at the molecular level, more clinical verification is needed to assess the application of these potential biomarkers (73). Tumor-educated platelets, which are believed to be able to accurately diagnose for various types of cancer in liquid biopsies, have recently been used as early diagnostic biomarkers for NPC. Wang *et al* (81) detected the expression of miR-34c-3p and miR-18a-5p in the platelets of patients with NPC and healthy controls, and found a reasonable diagnostic potential of the two miRNAs with a sensitivity of 92.59%, specificity of 86.11% and an area under the curve (AUC) value of 0.954. Although additional validation and functional analysis were not conducted, Best *et al* (86) pointed out that platelets, as anucleated cell fragments in the blood circulation, can modulate the splicing of their pre-mRNAs in response to signals from cancer cells and then change their transcriptome and molecular signals. Compared with other samples, platelets do not contain nuclei and are less interfered with by genomic DNA; therefore, their RNA expression truly reflects the pathological progression of tumors, providing a more valuable new platform for biomarker research (81).

Compared to PCR, microarray-based high-throughput technology has many advantages. However, it is often not sufficient to use only common features, such as gene names, for comparisons across different microarray platforms (7,8). The main reason for this is that different probes used in different studies may not have the tagging gene name or different probes of the same gene may not give similar signals (73). Therefore, it has been suggested that additional verification by PCR is necessary for the final identification of biomarkers in an independent verification cohort, as it allows the evaluation of gene expression more reliably (7). Wu *et al* (83) detected the miRNA expression profile in NPC saliva for the first time. They applied the stacking-hybridized universal tag (SHUT) miRNA array and quantitative PCR (qPCR) technology to compare the

miRNA expression levels in patients with NPC and healthy controls, and determined that the sensitivity and specificity of 12 differential miRNAs used to distinguish between patients with NPC and a healthy population were 100 and 96%, respectively. Bioinformatics analysis showed that these differentially expressed miRNAs play an important role in the development of NPC by regulating their target genes, such as platelet-derived growth factor receptor α ; *RAC1*; inhibitor of NF- κ B kinase regulatory subunit γ ; X-linked inhibitor of apoptosis; and protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1D. Furthermore, Li *et al* (87) reported the ability of hsa-mir-1281 and hsa-mir-6732-3p to evaluate the efficacy of RT for NPC by assessing the serum of patients with different RT sensitivities. Unsatisfactory AUC values of both miRNAs were found (0.750 for hsa-mir-1281 and 0.696 for hsa-mir-6732-3p); however the small sample size included in the study, as well as the fact that >50% of patients had stages III-IVa NPC and did not receive RT alone, may have affected the results.

Long non-coding (lnc)RNAs also play important roles at the transcriptomic level, performing a regulatory role in NPC epigenetics (88). Previous studies have confirmed that lncRNAs, miRNAs and EBV products can target each other and share common signaling pathways to form a complex molecular regulatory network (89). In recent years, novel lncRNAs have been identified through high-throughput sequencing to impact on the occurrence, progression, recurrence, metastasis and prognosis of NPC. However, the purpose of these studies was mainly to determine the mechanisms of action and treatment targets of NPC in different stages, rather than to identify biomarkers for NPC (88). He *et al* (90) used reverse transcription (RT)-qPCR to detect NPC-related lncRNAs in four different NPC cell lines and normal nasopharyngeal epithelial cells, as well as in the serum of patients with NPC, patients with chronic nasopharyngitis, EBV carriers and healthy controls. The AUC value of the three combined lncRNAs, including metastasis associated with lung adenocarcinoma transcript, actin filament-associated protein 1-antisense RNA1 and AL359062, to discriminate between patients with NPC and the healthy population, was 0.918. Furthermore, they found that the expression levels of these three lncRNAs significantly decreased in serum after treatment, thus confirming their potential as early diagnostic and efficacy evaluation biomarkers for NPC. Based on the current research progress, there is still a huge space for researchers to explore the value of lncRNAs as NPC biomarkers through liquid biopsy. High-throughput based second-generation sequencing technology does not rely on the prior knowledge of genomic information, which helps to identify new splicing points and mutations. Similar to miRNAs, an increasing number of lncRNAs have been found to be significantly related to the mechanism of action behind the radiosensitivity of NPC, but there are few clinical studies involving noninvasive detection (91).

Recently, RNA-sequencing has been used to detect the transcriptional profile of peripheral blood mononuclear cells in patients with NPC before and after RT. Although a study has reported that the 11 genes that have been found can be used as biomarkers to evaluate the prognosis of NPC after RT, a very small sample size was used and they did not establish a model for verification (92). In another study, Shuai *et al* (93) detected

increased expression levels of circular RNA_0000285 at the homeodomain interacting protein kinase 3 locus in NPC cells, tissues and serum samples, which could be used as a biomarker to predict the radiosensitivity of NPC. However, their study also lacked a diagnostic analysis of the sensitivity and specificity of biomarkers. It should be noted that the interaction test is a common statistical method to confirm the identified differential genes as potential biomarkers and this method is usually based on statistical models (94). Therefore, although several potential NPC biomarkers have been found in various studies, owing to the interference of multiple factors, there are still no widely verified and accepted biomarkers in the field of transcriptomics.

4. NPC biomarkers discovered through proteomics

Proteins are involved in many important physiological functions, such as immunity, coagulation, substance exchange, transportation, metabolism and signaling pathway regulation (95). Their composition and expression levels are often affected by the pathological state of the body (96). Proteomics is a research field that is based on the protein expression profile of the normal human body. Proteins with significant differences in expression levels can be identified by screening and comparing all of the proteins expressed in the cells, tissues or blood of individuals (97). With the assistance of continuous innovation of high-throughput technology, an increasing number of proteins related to the pathogenesis of diseases have been found, allowing for further elucidation of the pathogenesis behind various diseases and the identification of molecular biomarkers and novel drug targets (98).

Several studies have shown that gene mutations in cancer often cause abnormal expression levels of corresponding proteins and such expression can be dynamically changed following damage to the DNA of cancer cells following therapies (7,9). Furthermore, tumor cells can capture many protein decomposition products from normal tissues to synthesize the proteins they need, resulting in significant changes in protein expression levels due to increased anabolism and catabolism (99). Therefore, the proteins involved in carcinogenesis are considered functional molecules that can reflect the real-time state of disease progression and are used in the study of NPC biomarkers (7). Previous studies have identified various protein markers in different NPC cell lines, tissues and bodily fluid samples, including EBV-encoded proteins such as latent membrane protein (LMP)1, LMP2A and EBV nuclear antigens (9,25,99). Owing to the accumulated knowledge from these studies, the mechanism of protein action in each stage of NPC has been further clarified and multiple protein-protein interaction networks have been established based on various signaling pathways (100). This has laid a foundation for future research, based on the phosphorylation, ubiquitination, sulfation, methylation and sumoylation of NPC protein markers (101).

Compared with 2D fluorescence difference gel electrophoresis, isobaric tags for relative and absolute quantification; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; surface-enhanced laser desorption/ionization time of flight mass spectrometry; and other technologies used in earlier studies, proteomics has made significant progress

in the high-throughput technology of relative and absolute quantitation in recent years. For example, data-independent acquisition (used for comparative proteomics studies), parallel reaction monitoring (PRM) and phosphorylated PRM (used to study targeted proteomics), have improved significantly in the integrity of scanning, reproducibility, stability of results and the quantitative ability, and have been applied to study tumor biomarkers (7,9,25,102-105). However, studies on NPC proteomics based on bodily fluid samples have not employed these new technologies and have shown a decreasing trend in the study number compared with earlier studies (106-111). The reasons for this are not clear, but it may be related to the difficulty in obtaining samples, the equipment conditions of the laboratory and the cost of testing.

Recently, Sun *et al* (106) used RT-qPCR to detect the mRNA expression levels of insulin receptor substrate 1 (IRS-1) in 133 patients with NPC and 104 healthy controls. The sensitivity and specificity of IRS-1 in the early diagnosis of NPC were 88.0 and 77.9%, respectively. Coghill *et al* (107) applied a protein microarray to detect the anti-EBV-IgG and IgA antibody responses in 607 residents of Taiwan and found significant differences in the anti-EBV antibody levels of 60-IgA and 73-IgG between patients with NPC and healthy controls. The sensitivity and specificity of EBV VCA-IgA to distinguish patients with NPC were 66.7 and 95.0%, respectively, with an AUC value of 0.811. In another study, 2D gel electrophoresis, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and ELISAs were used to detect the serum auto-antibody levels of peroxiredoxin (PRDX)2 and PRDX3 in patients with NPC and CNE2 cells. Levels of PRDX2 and PRDX3 were significantly higher in patients with NPC and CNE2 cells than in the normal controls (108). In addition, Gong *et al* (109) detected cytokines in the serum of various cancer patients using antibody array technology. ELISA validation results showed that nine cytokines could be used as potential biomarkers for differential diagnosis and prognosis of NPC.

In terms of efficacy evaluation and prognosis, like genomics and transcriptomics, the evaluation of NPC radiosensitivity is still the main research focus of proteomics (17,110). NPC cells develop resistance to RT, which involves cell cycle regulation, apoptosis and anti-apoptosis, as well as DNA damage and repair, which may lead to the abnormal protein synthesis of NPC cells and affect the protein expression level in blood. This difference can be found by assessing the serum protein expression levels of RT-sensitive patients (7,9). Based on this hypothesis, Zhang *et al* (17) used the tandem mass tag method coupled with UPLC-MS/MS to detect the serum protein profile in patients with NPC with different RT effects. They found that the sensitivity and specificity of the combined five identified differential proteins, such as secreted protein acidic and rich in cysteine, serpin family D member 1S, complement C4B, peptidylprolyl isomerase B and family with sequence similarity 173 member A to distinguish patients with RT resistance, were 94.1 and 92.6%, respectively, and the AUC value was 0.968. In addition, since there is currently no effective predictor for NPC recurrence, Meng *et al* (110) used the same technique to compare the serum protein expression levels in patients with NPC who developed recurrence. The results of ELISA validation revealed that differentially

expressed calmodulin can be used as a potential biomarker for the diagnosis of NPC recurrence.

Although some researchers are still using bodily fluid samples to promote the research of NPC proteomics, the following obstacles need to be overcome to transform proteomics biomarkers into clinical applications. Initially, as mentioned above, the high-throughput technologies used in the existing studies are still relatively old. The limitations of these technologies in protein quantification, data collection, low abundance protein detection and repeatability restrict the discovery of some novel biomarkers with clinical significance (98,111). Moreover, most studies are not adequate with respect to the study design, which could affect the results by a variety of confounding factors, thus interfering with the sensitivity and specificity of the markers (94). For example, in the study of efficacy evaluation and prognostic biomarkers, researchers should focus on the dual effects of RT and CT on blood components, as well as the use of recognized and unified clinical efficacy evaluation standards and external data sets to fully evaluate and verify the results (112). Eventually, there is a significant difference in the application of the detection technology, pathological background of samples and statistical analysis tools, and the functional research often cannot be combined with clinical studies (113,114). These factors are bound to affect the repeated validation of identified biomarkers in different laboratories. In 2016, the US cancer moonshot 2020 officially launched a plan for precision medicine (115). Through the establishment of a data system for characterization of gene information and protein information, using genomics and proteomics as routine detection methods may provide more accurate guidance for individualized cancer treatment in the future. Therefore, it is believed that in the coming years, there will be an upsurge in NPC proteomics research based on high-throughput technology (116).

5. NPC biomarkers discovered through metabolomics

Briefly, metabolomics is a subject that conducts both qualitative and quantitative analysis of all low molecular weight metabolites (<1,000) of a certain organism or cell at the same time in a specific physiological period (117). It is based on high-throughput mass spectrometry technology, cluster index analysis and data processing, combined with information modeling and system integration, to screen and identify the differential metabolites correlated to the disease phenotype (118). Compared with other omics fields, metabolomics is rather new and has currently attracted substantial interest in the field of tumor biomarkers (119).

As downstream products of genes and proteins, bioactive metabolites are an important component of systems biology, because they can directly regulate biological processes and phenotypes by regulating the main mechanisms of action behind the functions of DNA, RNA and protein (117). Metabolic change is an important feature of cancer. To maintain continuous proliferation and survival after treatment, cancer cells must adjust their metabolism and nutritional needs (120). Metabolic disorders in cancer cells can further affect the expression of cell surface markers through a variety of functional signaling pathways (121). The study of metabolic phenotypes can help to find more reliable evidence for

the regulatory mechanism of the system that occurs or has occurred, rather than predicting the possible or upcoming changes. Therefore, metabolomics studies are not limited to the discovery of specific biomarkers. The exploration of characteristic pathological pathways and therapeutic targets can provide a more accurate direction for the development of novel medications (122,123).

Previously, a gas chromatography-mass spectrometry (GC-MS)-based metabolomics platform was the main technology employed to study bodily fluid samples. Tang *et al* (124) used this technology to detect 51 serum metabolites in 49 patients with NPC, 37 with laryngeal cancer and 40 healthy controls. Through the validation of differential metabolites in tissues and in the serum of patients with NPC, they found that kynurenine, N-acetylglucosaminylamine, N-acetylglucosamine and hydroxyphenylpyruvate can be used as potential biomarkers for the early diagnosis of NPC. Furthermore, they observed changes in these four metabolites at three time periods after RT and found that the high expression was closely related to cancer recurrence and distant metastasis, thereby confirming the efficacy and prognosis of these potential diagnostic markers. In another study, Yi *et al* (125) compared the serum metabolic profile in 100 patients with NPC to that in healthy controls and found that the sensitivity and specificity of seven metabolites (glucose, linoleic acid, stearic acid, arachidonic acid, proline, β -hydroxy butyrate and glycerol 1-hexadecanoate) for NPC diagnosis were 88.0 and 92.0%, respectively. Furthermore, they found that β -hydroxy butyrate and arachidonic acid can be used as evaluation indexes for the favorable prognosis of RT.

Along with the deepening understanding of NPC, the TNM staging system, RECIST and NCCN guidelines have changed significantly (12,23). Compared with early two-dimensional RT, the application of IMRT technology has significantly improved the treatment of NPC (126). Therefore, there is no need to evaluate the aforementioned studies in these aspects. However, it is important to ensure early studies have carried out long-term follow-up of patients, which may help to further verify the dynamic performance of the biomarkers. Compared with GC-MS, high-throughput, widely targeted metabolomics technology can solve the problem that non-targeted metabolomics methods cannot detect metabolites in batches, and may show higher sensitivity and high throughput performance in the known qualitative and quantitative analysis of metabolites, as well as in the detection of low-abundance metabolites. Therefore, it has been successfully applied to screen tumor biomarkers with the advantages of a self-built database to identify new metabolites (127,128). However, in recent years, to the best of our knowledge, there have not been any reports on the identification of NPC biomarkers in this field. This may also indicate the research potential and value of this field.

6. Key points for study design

Obtaining ideal biomarkers requires rigorous scientific study design and any design flaw will directly affect the experimental results and its clinical translational potential (94). Based on the different study purposes, the selection of case-cohort, bodily fluid samples and detection technology should be different,

and the influence of tumor stages, treatment methods and experimental conditions should also be considered (129).

In the selection of case-cohort, the study of early diagnostic markers should try to avoid the inclusion of patients with locally advanced or distant metastasis, because the secretion of metastatic tumors in cervical lymph nodes or distant organs cannot be ignored in biomarker screening (62,67,76,79-83, 106,107,109). Although the smaller number of patients with early-stage disease is the biggest obstacle to conducting such studies, their blood composition may provide feedback on the simplest specific marker information (5). Similarly, studies on biomarkers for NPC radiosensitivity should also focus on early-stage patients as most patients in stages III-IVa need additional CT (17,51,87). A previous study showed that the addition of cisplatin and paclitaxel can affect the expression levels of malondialdehyde, superoxide dismutase, catenin, glutathione, r-glutamyl cysteinyl and glycine in the blood of patients with NPC (130). Another study also confirmed that CCRT, RT or IC treatment may lead to varied changes in the distribution of metabolites in the serum of patients with head and neck cancer (131). Conversely, in a phase III clinical trial, Sun *et al* (112) found that the total IC efficacy of the combination of docetaxel, cisplatin and fluorouracil in the treatment of stages III and IV head and neck squamous cell carcinoma was significantly higher than that of the combination of cisplatin and fluorouracil (76.3 vs. 52.9%). However, this difference disappeared after CCRT (75 vs. 73.9%), indicating that RT may also have a significant effect on the efficacy of IC.

Serum and plasma are the most commonly studied bodily fluids (132). Several studies have found that compared with the coagulation of serum during collection, the miRNA-aligned reads produced in rat plasma during detection were twice as high as those produced by serum, but this difference was not found in human blood (133). However, among the 64 blood test indexes with statistical differences introduced by the WHO, 56 showed that the detection stability of plasma was significantly better than that of serum (134). Although this observation has been put into practice in several studies on the relationship between NPC and EBV, further verification and discussions are required to obtain general recognition (77,135). In addition, the collection, processing and storage of samples will have a significant effect on the experimental results (93). Taking the use of anticoagulants as an example, the protocol of sample handling and storage in the British biobank has clearly defined the scope of application of anticoagulants such as citrate, EDTA and heparin (136). Several other reports have also discussed the effects of various storage and processing methods on the composition of the blood (137,138). Thus, it can be seen that the normalization of procedures will help standardize the handling of samples in future studies and improve the quality of molecular detection.

For the detection technology, there is no doubt that new technology can often make up for the shortcomings of previous ones and that they are conducive to obtaining more specific biomarkers with respect to screening range and convenience of data analysis (139). Through a review of biomarker studies on the bodily fluid samples of patients with NPC in recent years, it is clear that there is a lack of application of new technologies in current studies, especially in the field of proteomics and metabolomics. Because of funding constraints, researchers

must often make a difficult choice between technology and sample size, which is the main reason most studies have difficulty in achieving authoritative recognition (140). In addition, although the screening and identification of biomarkers using bodily fluid samples have represented a complete story, it is still necessary to conduct cellular-based and molecular biology techniques to confirm the functions of these potential biomarkers.

7. Conclusion and future perspectives

Compared to the study of other common types of cancer, that of NPC is concentrated in Asian countries, specifically China, owing to the geographical distribution of its incidence (1,3,10). Great progress has been made in clinical trials of this disease and the NCCN guidelines have been rewritten (12). Conversely, biomarker research based on liquid biopsies is lagging, especially in the fields of proteomics and metabolomics, which limits the advancement of relevant research to a certain extent (7-9,13-15,17,100,124,125). For example, IC, as a standard initial method for systemic treatment of LA-NPC, the results of clinical trials show that not all patients can benefit from its use (15). Compared with CCRT alone, the prognosis of a considerable number of LA-NPC patients has not been significantly improved due to the addition of IC, indicating that they have suffered unnecessary over treatment (22). Conversely, in those who have extended the survival by IC+CCRT, 20-30% patients with LA-NPC still present tumor relapse and metastasis, suggesting that the current clinical application of IC may also provide insufficient treatment (13,14). Therefore, it is urgent to identify molecular biomarkers to predict the short-term efficacy of IC. In addition, because of serious toxic reactions in the blood, digestive tract, skin, nervous system, liver and kidney caused by RT and CT, new therapeutic targets are necessary for the development of new, safer medications (10,20,21). Therefore, future research on NPC biomarkers should gradually extend to the evaluation of efficacy and prognosis based on early diagnosis to provide sufficient laboratory data for the clinical research of NPC.

With the development of tumor biomarkers, it is impossible to fully explain the complex biological processes and network regulation behind the carcinogenesis and progression of tumors from a systematic perspective only by studying a single omics (141). Therefore, multi-omics integration has become a new trend to promote the research and development of tumor biomarkers (142). The pathogenesis behind the changes required for cancer, gene mutation, transcriptional regulation, protein synthesis and metabolic changes constitute a systematic mechanism (143). Based on these relationships, the integration of two or more kinds of omics research and the use of machine learning methods to carry out association analysis on molecules at multiple levels, could make up for the lack of data caused by single omics analysis and reduce the probability of false positive results. This would allow researchers to study the phenotype and regulatory mechanisms of action in biological models more effectively and investigate complex scientific problems more comprehensively. By using this strategy, NPC biomarkers may effectively compensate for the deficiencies in early omics research, promoting the overall development of NPC clinical research.

Collectively, novel clinical issues, scientific study design, cutting-edge high-throughput technology, integrated multi-omics platforms, extensive screening of large-scale cohort, systematic functional analysis, ensemble learning and comprehensive and in-depth validation are inevitable methods that are required to enhance research into NPC biomarkers using bodily fluid samples.

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Authors' contributions

JCL and SQZ conceived and designed the review. SQZ, SXL and YSH consulted the literature. SQZ analyzed the literature and drafted the manuscript. YSH produced the figure. JCL, SMP and HBC critically revised the article for important intellectual content and assisted in the literature search for this review article. All authors read and approved the final version of this manuscript.

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Competing interests

All the authors declare that they have no competing interests.

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