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Review

Circulating nucleic acids as a tumor marker

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Summary. Patients suffering from malignant diseases have been shown to have increased amounts of cell free nucleic acids in their circulation. As genetic and epigenetic alterations are increasingly characterized in different types of tumors, such changes can be used to detect tumor-derived nucleic acids in the circulation. To date, nearly all tumor-associated nucleic acids have been detected in the plasma or serum of cancer patients. Moreover, increased levels of circulating viral nucleic acids have also been demonstrated in patients with certain cancers associated with viral infections. The concentration of these tumor-associated nucleic acid species is generally related to the tumor load and the extent of the disease. Serial monitoring of plasma nucleic acids thus provides a good way to follow disease progress and to predict the outcome of such patients. In this review, different approaches of detecting tumor-related nucleic acids in the circulation and their potential as tumor markers in the screening, monitoring and prognostication of malignant diseases are discussed.

Key words: Circulating nucleic acids, Tumor markers, Cancer, Plasma/serum DNA

Introduction

Despite much improvement in cancer therapy in the past decades, early diagnosis is still the key to successful curative treatment of cancer. Tumor markers have therefore been developed for the purpose of screening for early malignant disease. Besides, they are also valuable in monitoring cancer progression as well as prognostication. Existing tumor markers are mainly proteins. Many of them can be quantified by immunoassays. However, only a subset of cancers would secrete specific proteins that can be used as tumor markers. With the recent developments of molecular biological techniques, multiple genetic and epigenetic alterations have been characterized in tumors. Of diagnostic significance is the fact that many of such changes are also detectable in the plasma or serum of cancer patients. This opens up the potential of utilizing circulating nucleic acids as a new generation of tumor markers.

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History of plasma nucleic acids

The first discovery of circulating nucleic acids was reported by Mandel and Metais in 1948 (Mandel and Metais, 1948). They demonstrated that extracellular DNA and RNA could be detected from the blood of healthy as well as sick individuals. This report was published only a few years after the demonstration that DNA was the material of inheritance, and even preceded Watson and Crick's classical paper on the double helical structure of DNA (Watson and Crick, 1953). However, their pioneering work had not aroused much interest at that time. Further development of the field had to await some 30 years until further technical advances were made. In 1977, Leon and Shapiro showed that cancer patients had higher concentrations of serum DNA than those suffering from nonmalignant diseases (Leon et al., 1977). In some of these patients, the level of circulating DNA decreased after successful anticancer therapy (Leon et al., 1977). Owing to the technological limitation at that time, the precise cellular origin of the extracellular DNA in cancer patients could not be determined.

In 1989, Stroun et al. showed that the DNA found in plasma of cancer patients carried certain neoplastic characteristics (Stroun et al., 1989). In 1994, two groups of investigators demonstrated that specific oncogene mutations could be detected in the plasma of patients suffering from pancreatic cancer (Sorenson et al., 1994) and myelodysplastic syndrome or acute myelogenous leukemia (Vasioukhin et al., 1994). This was followed up by other studies based on microsatellite analysis in 1996. Loss of heterozygosity (LOH) and microsatellite instability were found in the primary tumors as well as the plasma or serum of patients suffering from lung

cancer (Chen et al., 1996) and head and neck cancer (Nawroz et al., 1996). These findings suggested that high fractional concentrations of tumor DNA were present in the plasma/serum DNA of these patients.

To date, many tumor-associated genetic and epigenetic changes have been detected in the plasma and serum of cancer patients. Moreover, increased levels of circulating viral nucleic acids have been demonstrated in patients with cancers associated with viral infection (Lo et al., 1999a,b). In this review, different approaches of detecting tumor-related nucleic acids in the circulation and their potential as tumor markers in the screening, monitoring and prognostication of malignant diseases are discussed.

Circulating oncogene mutations

Oncogene mutations can be found in a wide variety of cancers. The detection of such mutations in plasma/serum provides concrete evidence that cell-free nucleic acids are released into the circulation by tumors. Mutated *RAS*-gene was the first tumor-specific sequence detected in the blood of cancer patients. In 1994, Sorenson et al. detected mutated *KRAS* sequences in the serum of patients with pancreatic tumors (Sorenson et al., 1994). At the same time, Vasioukhin et al reported the presence of point mutations of the *NRAS* gene in the plasma of patients with myelodysplastic syndrome or acute myelogenous leukemia (Vasioukhin et al., 1994). Among different oncogenes, plasma *RAS* gene mutations are of particular interest and are most extensively studied. First, they are frequently found in various types of tumors, including carcinoma of the pancreas, colorectum, lung and thyroid. Second, the presence of point mutations in the *KRAS* gene in the plasma/serum of patient is frequently associated with cancer. Furthermore, the relatively limited repertoire of *KRAS* gene mutations makes its detection practical (Sorenson, 2000).

Most studies concerning the *KRAS* mutations in plasma/serum have been performed in patients suffering from colorectal and pancreatic tumors because these tumors carry relatively high frequencies of *KRAS* mutations. *KRAS* mutations can be detected in around half of the tumor tissues of colorectal carcinoma (Morrin et al., 1994; Smith-Ravin et al., 1995). However, the frequency of detecting mutated *KRAS* sequences in plasma/serum in patients with colorectal cancers is highly variable. Several groups reported that mutated *KRAS* sequence could be found in over 80% of the patients' plasma/serum (Anker et al., 1997; de Kok et al., 1997; Kopreski et al., 1997). However, Hibi et al. detected mutated *KRAS* sequence in only 19% of plasma samples from such patients (Hibi et al., 1998). In view of the small sizes of these studies, no significant correlation can be observed between the presence of *KRAS* mutations in plasma/serum and the clinicopathological status of the patients.

Results on pancreatic cancer patients are less ambiguous. In approximately half of the patients, mutated *KRAS* sequences can be detected in their plasma/serum (Sorenson et al., 1994; Mulcahy et al., 1998; Yamada et al., 1998; Castells et al., 1999). These patients generally have higher clinical stages, larger tumors and are more likely to have metastasis. Moreover, those patients with positive assay results before treatment are less likely to result in a cure after surgical resection (Yamada et al., 1998). Circulating mutated *KRAS* sequences can also supplement existing assay of CA19-9 for screening of pancreatic carcinoma since it is positive in half of the patients with relatively low CA19-9 levels (Yamada et al., 1998). The presence of mutated *KRAS* sequences in blood may even precede the diagnosis of pancreatic carcinoma by 5-14 months (Mulcahy et al., 1998). It is of interest to note that mutated *KRAS* sequences are also detected in the circulation of a small proportion of patients suffering from chronic pancreatitis (Mulcahy et al., 1998).

Apart from *RAS* gene mutations, mutated *p53* sequences and *APC* sequences have also been detected in the plasma or serum of patients with colon, breast and lung cancers (Mayall et al., 1998; Silva et al., 1999; Gocke et al., 2000; Gonzalez et al., 2000). The presence of these mutated genetic sequences in plasma/serum is generally associated with more advanced staging and larger tumors. When present in plasma/serum, these oncogene mutations usually correspond to those found in the primary tumor. In addition, the presence *p53* gene mutations in plasma was also shown to be an independent prognostic factor for recurrence and metastasis of breast cancer after treatment (Shao et al., 2001).

With the aid of more accurate quantification techniques, amplifications of oncogenes and deletions of tumor suppressor genes can be demonstrated in tumor tissues. Chiang et al. showed that in patients suffering from carcinoma of the esophagus, amplifications of the *erbB-2* gene could also be detected in their serum (Chiang et al., 1999).

Microsatellite alterations

Microsatellite instability (MSI) and LOH are frequently demonstrated in tumor tissues. The detection of LOH in plasma/serum was simultaneously reported by two groups in 1996 in patients suffering from small cell lung carcinoma and head and neck squamous cell carcinoma (Chen et al., 1996; Nawroz et al., 1996). The changes found in plasma/serum were identical to those found in the primary tumors. This finding not only confirmed the presence of tumor DNA in plasma/serum and further suggested that they constituted a major proportion of the circulating DNA.

LOH has been detected in the plasma/serum of patients having carcinoma of the lung, head and neck, breast and melanoma. As different groups of investigators have used different numbers and locations of microsatellite markers, their results are very

heterogeneous. The detection rate of LOH and MSI in the plasma of cancer patients varies from 2% to 71% (Coulet et al., 2000; Gonzalez et al., 2000). When present, these changes usually correspond to those found in the primary tumors. Positive results can be seen in patients having small tumors or in situ carcinomas (Chen et al., 1999; Nunes et al., 2001). This indicates that the amount of DNA released from the tumor can be substantial even in the early stage of the disease. On the other hand, there is a trend that positive results correlate with the clinicopathological characteristics of the patients. Patients having LOH in their plasma/serum are more likely to have invasive tumors, regional spreads and distant metastases (Fujiwara et al., 1999; Silva et al., 1999; Gonzalez et al., 2000; Taback et al., 2001). The presence of LOH at microsatellite markers in plasma in melanoma patients has been shown to be a bad prognostic factor (Taback et al., 2001).

Sozzi et al. and Gonzalez et al. further demonstrated that in patients whose plasma showed LOH, such change would disappear from their plasma after successful treatment (Gonzalez et al., 2000; Sozzi et al., 2001). On the other hand, in patients with no response to treatment, normalization of plasma DNA abnormalities could not be seen. These tumor-specific changes reappeared later in the responding patients with the recurrence of the disease (Gonzalez et al., 2000). Therefore, microsatellite markers seem to be valuable in the prognostication as well as monitoring of tumor progression.

Viral nucleic acids

Virus infection has been implicated in the pathogenesis of several types of cancers. This relationship suggests that circulating viral nucleic acids may be a potentially useful tumor marker for some of these tumors. The most exciting example is the presence Epstein-Barr virus (EBV) DNA in the plasma/serum of patients suffering from nasopharyngeal carcinoma (NPC). Since the level of circulating EBV DNA in NPC patients corresponds well to their disease status, this assay holds much promise to be incorporated into clinical practice for the early detection and monitoring of disease.

EBV appears to be a very important etiological factor for NPC (Liebowitz, 1994). The viral genome is detectable in almost all NPC tissues and is usually clonal in origin. With the use of real-time polymerase chain reaction (PCR) technology, Lo et al were able to detect circulating EBV DNA in 96% of NPC subjects (Lo et al., 1999). In addition, a correlation between the concentration of plasma/serum EBV DNA and disease stage was observed (Lo et al., 1999). Patients with advanced stage disease had a higher plasma/serum EBV DNA than those with early disease. The latter observation suggests that the concentration of plasma/serum EBV DNA may be a reflection of the tumor load in a particular patient. Moreover, Lo et al. have also found that plasma/serum EBV DNA is an independent prognostic factor of disease stage for long-term survival (Lo et al., 2000).

Following radiotherapy, plasma/serum EBV DNA has been found to be a valuable marker for post-treatment monitoring (Lo et al., 1999). In most patients, plasma/serum DNA level dropped after radiotherapy, following an initial surge (Lo et al., 2000). However, the persistence of plasma/serum EBV DNA at one month following the completion of radiotherapy was shown to be a bad prognostic sign (Lo et al., 1999). Serial monitoring of patients indicated that patients who had undetectable or a low level of EBV DNA remained in continuous clinical remission (Lo et al., 1999). On the contrary, in most patients with tumor recurrence, a rise in plasma/serum EBV DNA could be observed. With the success of correlating plasma/serum EBV DNA level to the clinical course of NPC, Lo et al also showed that mRNA transcripts from the EBV genome are also detectable in the plasma of NPC patients (Lo et al., 1999).

As human papilloma virus (HPV) is a crucial etiological factor for cervical carcinoma and some head and neck squamous cell carcinoma, the possibility of using HPV DNA as a marker for these cancers has also been explored. In contrast to the high frequency of detecting EBV DNA in NPC patients' plasma/serum, the detection rate of HPV DNA in plasma/serum of cervical cancer and head and neck cancers patients is only around 10% (Capone et al., 2000; Pornthanakasem et al., 2001). HPV DNA can only be detected in the plasma of patients with more advanced disease and is associated with the presence of metastasis. As HPV DNA cannot be found in the plasma/serum of control subjects or HPV-negative cervical cancer patients, this highly specific assay is thus valuable as a marker for metastasis or advanced disease.

Why is there such a great discrepancy between the detection rate of EBV in NPC and HPV in cervical and head and neck cancers? The mechanism of how viral DNA is released into the circulation of cancer patients is still unclear. It has generally been believed that in NPC patients, EBV exists in a latent state. However, Shotelersuk et al showed that at least a proportion of the EBV DNA found in the plasma/serum of NPC patients is resistant to DNase digestion (Shotelersuk et al., 2000). These data suggest that intact viral particles, instead of free naked nucleic acids, may be present in the circulation of NPC patients. On the other hand, HPV DNA detected in plasma/serum of cervical cancer patients shows deletion in the E2 gene and suggests that these viral genomes have gone through the process of integration (Pornthanakasem et al., 2001). These findings suggest that the exact molecular events involving a certain virus in a particular type of tumor may influence the ultimate suitability of such viral nucleic acid as a plasma/serum marker.

Epigenetic changes

Epigenetics describes the inheritance of information

by the modifications of DNA other than its sequence, in contrast to genetics, which describes the inheritance of information on the basis of DNA sequence. DNA methylation is an epigenetic characteristic associated with the silencing of gene expression. Alterations of DNA methylation patterns, including global genome hypomethylation and regional hypermethylation of tumor suppressor genes or their promoters, are increasingly found in different types of tumors. These changes are thought to be closely associated with tumorigenesis. Genes which are frequently hypermethylated in multiple tumors, include *p15* (Herman et al., 1996), *p16* (Herman et al., 1995), *APC* (Kawakami et al., 2000), *BRCA-1* (Esteller et al., 2000), *E-cadherin* (Graff et al., 1995), *LKB1* (Esteller et al., 2000), *VHL* (Herman et al., 1994), *hMLH1* (Herman et al., 1998) and *MGMT* (Esteller et al., 1999). Many of these genes play important roles either in the regulation of cell cycle or in the pathway of DNA repair (Baylin and Herman, 2000). Development of methods like methylation-specific polymerase chain reaction (MSP) (Herman et al., 1996) allows us to detect small amounts of hypermethylated sequences in a background of wide type sequences and thus makes detection of such sequences in plasma/serum of cancer patients possible.

The *p16* and *p15* genes code for two cyclin dependent kinase inhibitor proteins which are important in the regulation of the cell cycle (McDonald and El-Deiry, 2001). Hypermethylation of the promoter regions of these genes have been reported in many types of tumors (Herman et al., 1996). These epigenetic changes can be detected in the tumor tissues of all disease stages (Wong et al., 2000a-c; Hibi et al., 2001). This is consistent with the hypothesis that hypermethylation of these genes is an early event in tumorigenesis.

Recently, several groups have also reported the detection of these epigenetic changes in the plasma/serum of patients with carcinoma of the lung, esophagus, liver, breast and leukemia (Esteller et al., 1999; Silva et al., 1999; Wong et al., 1999, 2000a-c; Hibi et al., 2001). The rate of detecting such changes in plasma/serum depends on the sensitivity of the detection method. With MSP, hypermethylation of either the *p15* or *p16* gene can be detected in the plasma/serum in as much as 92% of hepatocellular carcinoma patients carrying such changes (Wong et al., 2000). In patients suffering from leukemia, *p15* methylation status shows a good concordance with the morphological disease stage and the early reappearance of such changes during remission predicts relapse (Wong et al., 2000a-c). Nonetheless, the presence of such epigenetic changes does not show any significant association with the clinicopathological characteristics of the patients with solid tumors (Esteller et al., 1999; Wong et al., 1999; Hibi et al., 2001). This may be due to the small patient numbers in these early studies.

Detection of the hypermethylated *APC* gene in the plasma of patients suffering from esophageal adenocarcinoma has been shown to be significantly associated with reduced patient survival (Kawakami et al., 2000). Presence of aberrant methylation of the *DAP-kinase* gene in the plasma of head and neck cancer patients has been shown to be associated with lymph node involvement and advanced disease stage (Sanchez-Cespedes et al., 2000). Although the presence of these tumoral epigenetic changes in the plasma/serum is quite specific, the detection rate of any single marker is rather low. Therefore, the combination of multiple markers has been studied and shown to increase the sensitivity of detection. The presence of hypermethylation of at least one of the *MGMT*, *DAP-kinase*, *GSTP1* and *p16* genes can be detected in 73% of the serum of non-small cell lung cancer patients (Esteller et al., 1999).

Wong et al. have also demonstrated the relationship of *p16* methylation status and serum α-fetoprotein concentration in hepatocellular carcinoma (HCC) patients (Wong et al., 2000a-c). In patients with elevated α-fetoprotein levels, methylated *p16* sequences are generally detectable in their plasma/serum. Furthermore, these tumor-specific sequences could also be detected in the plasma of HCC patients with a low α-fetoprotein level (Wong et al., 2000a-c). Therefore, combining this assay with the α-fetoprotein assay can increase the sensitivity of the screening tests.

Mitochondrial DNA

Mutations in the mitochondrial genome have recently been documented for a number of cancers (Penta et al., 2001). Very recently, detection of mutated mitochondrial DNA sequences in plasma has been reported (Jeronimo et al., 2001). Jeronimo et al. studied 16 patients suffering from prostate cancer. They reported that identical mutations of the mitochondrial DNA could be detected in the plasma and tumoral tissues of three patients (Jeronimo et al., 2001). This study shows for the first time the presence of tumor-specific mitochondrial DNA in the circulation. As tumoral tissues contain high copy numbers of mitochondrial DNA sequence, detection of tumor mitochondrial DNA may offer a sensitive way to detect early disease.

RNA

mRNA is the intermediate between the coding gene and the final product, protein. The level of mRNA level in a tissue reflects the concentration of the respective protein. As different types of tumor typically express a different repertoire of protein, the respective mRNA can be used as a marker for cancer screening and monitoring. However, it has been known that RNase is present in the blood of healthy individuals and its level is increased in cancer patients (Reddi and Holland, 1976). Therefore, all free RNA in the blood would be expected to be rapidly destroyed. Nonetheless, in 1999, Lo et al. showed that EBV RNA could be detected in the blood of NPC patients (Lo et al., 1999). At the same time, Kopreski et al demonstrated the presence of tyrosinase mRNA in the

serum of one third of patients with malignant melanoma (Kopreski et al., 1999). In the same study, no tyrosinase mRNA could be detected in the serum of healthy subjects (Kopreski et al., 1999). Kopreski et al. also showed that these mRNA species were still detectable even after the serum samples had been passed through a 0.45 μm filter. These studies suggested that there were extracellular RNA molecules present in the circulation of cancer patients, possibly protected from RNase (Kopreski et al., 1999; Lo et al., 1999).

Several groups have confirmed these findings by demonstrating different types of mRNA in the plasma/serum of cancer patients. Chen et al. showed that telomerase RNA could be detected in the plasma of 44% of breast cancer patients (Chen et al., 2000). Using quantitative real time reverse transcriptase PCR, Dasi et al further showed that the mRNA levels of telomerase reverse transcriptase were significantly higher in patients with lymphoma and colorectal cancer than in healthy subjects (Dasi et al., 2001). Moreover, Silva et al. showed that the presence of epithelial mRNA in the plasma significantly correlated with tumor size and proliferative index (Silva et al., 2001). Detection of 5T4, a transmembrane glycoprotein, mRNA has also been reported to be present specifically in the serum of lung cancer patients (Kopreski et al., 2001).

Conclusions

Genetic and epigenetic alterations are increasingly detected in different types of tumors. To date, nearly all the changes present in tumor tissues are shown to be detectable in the plasma/serum of cancer patients. This observation can also be applied to those tumors closely associated with viral infections, like NPC which frequently harbors EBV DNA. The amount of these tumor-specific nucleic acid sequences is generally related to the tumor load and the extent of the disease. Monitoring the change in the constituent of the plasma nucleic acids provides a good way to monitor disease progress and to predict the outcome of the patients. A number of such plasma nucleic acid molecules have been shown to hold promise for routine clinical use. We can foresee that in the near future, more and more such markers will be developed, with sensitivity and specificity comparable, if not superior to the present protein-based assays.

Although the exact mechanism of how tumor DNA is released into the circulation remains unclear, reports have suggested that apoptosis may play an important role. Jahr et al. and Giacona et al. showed that the majority of DNA molecules present in plasma/serum have lengths in multiples of nucleosomal DNA, which is a characteristic of apoptotic cell death (Giacona et al., 1998; Jahr et al., 2001). The level of circulating nucleic acids in cancer patients is much higher than that of healthy individuals. However, not all the excess nucleic acids are tumor-derived (Jahr et al., 2001). Investigation of the mechanism of how normal nucleic acids are liberated into the plasma of tumor patients may improve our understanding of tumor biology.

The concentration of circulating nucleic acids varies greatly in healthy subjects and cancer patients. Different protocols of plasma processing have been shown to affect the yield, as well as the composition of nucleic acids (Chiu et al., 2001). As quantitative methods are increasingly used in the evaluation of tumor-specific sequences, standardization for both the material used and the preparation procedure is necessary so that reports from different groups would be comparable. With such developments, we are optimistic that circulating nucleic acids will add to our armamentarium for the combat against cancer.

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