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Exosomal microRNA Biomarkers: Emerging Frontiers in Colorectal and Other Human Cancers

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Abstract

Diagnostic strategies, particularly non-invasive blood-based screening approaches, are gaining increased attention for the early detection and attenuation of mortality associated with colorectal cancer (CRC). However, the majority of current screening approaches are inadequate at replacing the conventional CRC diagnostic procedures. Yet, due to technological advances and a better understanding of molecular events underlying human cancer, a new category of biomarkers are on the horizon. Recent evidence indicates that cells release a distinct class of small vesicles called ‘exosomes’, which contain nucleic acids and proteins that reflect and typify host-cell molecular architecture. Intriguingly, exosomes released from cancer cells have a distinct genetic and epigenetic makeup, which allows them to undertake their tumorigenic function. From a clinical standpoint, these unique cancer-specific fingerprints present in exosomes appear to be detectable in a small amount of blood, making them very attractive substrates for developing cancer biomarkers, particularly noninvasive diagnostic approaches.

Introduction

Colorectal cancer (CRC) remains the third leading cause of cancer-related deaths in the United States among men and women [1]. Since approximately 70% of deaths from CRC occur between the ages of 50–79, early detection and robust screening for CRC could significantly reduce mortality associated with this disease. A conscious effort in this regard has led to improvements in compliance for CRC screening (colonoscopy and fecal occult blood test (FOBT)), which has resulted in a 3% decrease in CRC incidence rates from 2000–2010 [1]. Even though early detection of adenomatous polyps or early-stage CRCs is often curable, only ~50% of the population between the ages of 50–64 in the United States follow

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recommended guidelines for CRC screening [1]. Currently, for the screening of colorectal adenomas and cancers, colonoscopy still remains the gold standard and has led to a reduction in mortality rates by 67% [2,3]. However, the challenge remains that in spite of its clinical significance, colonoscopic screening is not practical for everyone as it requires extensive pre-procedure preparation and due to its invasive nature. Not surprisingly, colonoscopic screening has yielded poor patient compliance in the general population, particularly within the age group of 50–65, who must follow recommended guidelines for CRC screening once they turn 45–50 years old [1]. Currently, FOBT and fecal DNA tests (FDT) are two approaches used for non-invasive screening of CRC. Although both methods contribute towards reduction in CRC mortality, low specificity and sensitivity rates have hampered their clinical utility for the identification of pre-malignant neoplasms [4]. This limitation is further compounded by additional factors such as the timing of screening, and the consistency of fecal matter that can negatively influence the detection rates of these assays [5]. In addition, participation in fecal screening tests has been generally suboptimal due to the negative perception associated with at-home fecal specimen collection, handling, and its transportation to the clinic for analysis. Not surprisingly, there is an overwhelming preference for blood-based vs. fecal screening [6], highlighting the need and potential merit for developing highly robust non-invasive blood-based tests for identifying colorectal polyps and early-stage cancers.

Analysis of nucleic acids, both RNA and DNA, in blood is currently being exploited aggressively for developing such diagnostic biomarkers. Extracellular vesicles are evolutionally conserved membrane bound organelles released by cells, and are postulated as one of the major contributors for the overall composition of nucleic acids in blood [7]. Conventionally, extracellular vesicles are divided into three main categories: 1) microvesicles/microparticles/ectosomes 2) exosomes 3) apoptotic bodies [8]. In particular, one of the very latest concepts that is gaining momentum is analyses of molecular cargo packaged inside endocytic membrane-derived vesicles called ‘exosomes’. Exosomes are approximately 30–140 nm in size and are released from various cell types [9]. It might seem counterintuitive that since so many cells release exosomes, these vesicles may not be ideal carriers of genomic information for biomarker discovery. However, a distinct and significantly important feature of exosomes is that they very efficiently carry and deliver molecular signatures (e.g. nucleic acids and proteins) from their ‘cell-of-origin’, to the ‘target-cell’, and this signal can be readily detected in blood. In other words, compared to free circulating biomarkers, exosomal constituents may provide the much needed layer of specificity for development of tissue/organ-specific biomarkers. Besides their diagnostic potential, early studies have revealed that exosomal content can reflect disease status and treatment response in human malignancies [10–12]. However, despite the rapid expanding interests in exosomal research and their promising potential as a cancer diagnostic marker, there are several well-recognized technical limitations including standardization of specimen handling, appropriate normalizers and isolation techniques [13]. Nevertheless, the overall positive factors far out-weigh these technical limitations and make exosomes attractive substrates for disease biomarker development.

One of the key molecular constituents extensively characterized within exosomal cargo to date has been non-coding RNAs, particularly microRNAs (miRNAs). MiRNAs are small

single-stranded RNAs known to regulate the expression of more than 50% of protein-coding genes through interactions with their target mRNA transcripts [14], and dysregulated expression of specific miRNAs is currently being explored as potential biomarker strategy in human cancers [15,16], including CRC [17–19]. Since the expression of most miRNAs in serum is generally low, whether we can successfully identify superior exosomal miRNA biomarkers is somewhat debatable and remains a topic of active investigation. This article will focus on the functional role of exosomes in cancer, and their potential as CRC-specific biomarkers in conjunction with the expression level of miRNAs.

Current Generation of Colorectal Cancer Biomarkers and Their Potential Limitations

The definition of a biomarker is *any biologically measurable substance that is indicative of a disease process, biological change, or infection*. Despite a plethora of potentially promising blood-based CRC biomarkers discovered over the years, the majority of these have not been successfully validated for their clinical use, since they have not been robust enough to justify replacing the current generation of clinical assays. Although a few other noninvasive screening tests that can detect traces of blood (or heme) in stool of cancer patients are commercially available, such as guaiac-based fecal occult blood tests (gFOBT) and fecal immunochemical tests (FIT), they have only resulted in a modest, 15–25%, reduction in CRC mortality [20]. While most studies contend that FIT has superior detection rates vs. FOBT [21], this approach has substantially lower detection rates for detecting colorectal adenomas (both benign and advanced) compared to colonoscopy [22].

In contrast to stool-based assays, it is generally believed that a blood-based test may be a more practical choice from a compliance perspective, since blood samples are routinely collected during clinic visits, including annual health examinations. A survey based study, targeting the age group of 50–74, showed an overwhelming preference for blood-based CRC screening over fecal assays [6]. Undoubtedly, technological advances will very likely improve the accuracy of fecal-based approaches in the future; nonetheless, it would still be an uphill task to alter the negative connotation associated with fecal-based tests. Therefore, development of robust non-invasive blood-based screening methods for CRC screening will have enormous clinical implication for early detection of the disease, and will also have significant impact on improving participation and compliance among general population.

Conventional Molecular Cancer Biomarkers

In order to identify potential candidates for blood-based cancer biomarkers, it is critical to understand how cancer cells release traces of DNA and RNA in the circulation. Initially, communication within the tumor microenvironment was believed to occur by simple diffusion of proteins, hormones, nucleic acids, and ionic movement. However, more recent evidence suggests that in reality, it is the specific set of molecules that are secreted by cancer cells that are responsible for inducing cancer progression and metastasis through a multitude of autocrine, endocrine, and paracrine mechanisms [23]. Increased accumulation and diffusion of these cellular constituents results in leakage into the circulation facilitating their detection in blood (as serum or plasma) in various downstream diagnostic applications [24]. One example of protein-based cancer detection markers is the prostate specific antigen (PSA), which is frequently being used clinically for screening patients with prostate cancer.

Unfortunately, now we recognize that high PSA levels are not the best indicators of prostate cancer, as its level can be elevated due to a variety of other clinical conditions, even in the absence of prostate cancer [25]. Similarly, hormones secreted by cancers resulting in paraneoplastic syndrome have been targeted as potential cancer biomarkers, but lack of cancer-specificity and high inter-individual variability associated with these hormones makes them undesirable candidates [26].

Over the years, circulating protein-based biomarkers have been explored for the early detection of CRC. In particular, carcinoembryonic antigen (CEA) is a well-established protein marker clinically used to monitor therapeutic response and tumor recurrence in cancer. High CEA levels were first discovered 50 years ago in both fetal colon and colonic adenocarcinomas, while significantly lower levels were noted in healthy adults [27,28]. Although CEA was considered as a promising diagnostic marker for CRC, unfortunately it is not readily detected in early stages of cancers [29]. Another potential protein-based CRC diagnostic marker is the epithelial cell adhesive molecule (EpCAM), a glycosyl phosphatidyl inositol (GPI) cell surface anchored glycoprotein, which mediates epithelium-specific intercellular adhesion. While EpCAM can be present in normal epithelium in a variety of tissues, it is particularly expressed at higher levels in the colon [30]. Intriguingly, EpCAM expression is often elevated in adenomatous tissues as well, which can also be detected in the serum [31]. However, EpCAM is also expressed in several other epithelial cancers - diminishing its specificity as a CRC-specific biomarker.

More recently, owing to their very high frequency and important functional relevance, cancer-associated epigenetic changes in DNA and RNA have been extensively interrogated for their potential as cancer biomarkers. In particular, DNA methylation is one of the most studied epigenetic alterations in cancer. Aberrant DNA methylation affects gene expression through hypo- and hyper-methylation of CpG islands within gene promoters and in the context of large repetitive sequences (e.g. centromeres or retrotransposon elements such as LINE-1, SINE-1/Alu sequences). A large panel of methylated genes has been explored as potential blood-based biomarkers for CRC, including *SEPT9* and *ALX4* [32,33]. However, low levels of circulating DNA in patient samples combined with the disease heterogeneity, has made development of such DNA methylation-based diagnostic approaches clinically challenging [34]. In addition, identification of aberrant gene methylation is particularly favorable during later stages of CRC, limiting the number of potential genes that can be used for the early detection of the disease [35]. In contrast, non-coding RNAs, such as miRNAs, are more easily detectable in serum, and appear to have a greater biomarker potential vis-à-vis DNA methylation markers in liquid biopsies.

Emerging evidence indicates that within the tumor microenvironment, cells communicate by releasing small vesicles called “exosomes”, and these molecular entities are gaining traction as potential disease biomarkers. The majority of past attempts aimed at the discovery of non-invasive cancer markers were primarily focused on contents within serum or plasma. However, recognition of exosomes and their unique characteristics provide an attractive avenue for development of more accurate diagnostic milieu released by the cancer cells.

Exosomes: New Frontiers in Cancer Biology

Exosomes are small phospholipid bilayer nano-vesicles excreted by a variety of cell types [36] including: dendritic cells, epithelial cells, endothelial cells, mast cells, lymphocytes, platelets, neurons, and most importantly cancerous cells [37–40]. Exosomes have been isolated from various bodily fluids such as blood [41], saliva [42], urine [9], amniotic fluid [43], inflammatory ascites [44], breast milk [45], and cerebrospinal fluid [46]. During their initial discovery in 1981, exosomes were first characterized primarily based upon two different size populations — either 40 or 500–1000 nm in diameter, which were incorrectly perceived to be “cell fragments” released into the cell culture media [47]. Identification of these fragments in subsequent studies investigating the function of reticulocytes [48] revealed that the 40 nm vesicular fraction of these cell fragments contained transferrin receptors, and were henceforth named as “exosomes”.

Even though various cell types secrete exosomes, their molecular composition appears to be cell dependent — making them particularly useful for characterization of specific tissue and cellular sub-types [36,49,50]. Their unique molecular profiles, as well as the size of exosomes, are used to distinguish exosomes from other microvesicles, which tend to be 140–1000 nm in size [51]. There are already several well-established surface protein markers including CD63, CD81, CD9, TSG101, and ALIX to identify exosomes [52]. Recent data indicates that a large variety of proteins and nucleic acids, including mRNAs and miRNAs, are packaged into exosomes, which contribute to various disease pathologies including: autoimmune disorders [42], viral replication [53], and human cancers [10]. Furthermore, proteomics-based characterization of exosomes has identified the presence of functionally relevant surface proteins such as flotillin-1, GPI-anchored proteins, and tyrosine kinases [54–56].

Biogenesis of Exosomes

Before we delve into the potential applications of exosomes as cancer biomarkers, it is important to understand how these tiny cell signaling powerhouses are generated. The biogenesis of exosomes consists of initiation, endocytosis, formation of multi-vesicular bodies (MVBs) and secretion as illustrated in Figure 1. Exosomal biogenesis initiates through the invagination of endocytic vesicles on the plasma membrane through the formation of early endosomes. The phospholipid bilayer of the early endosomes later folds to form multiple intra-luminal vesicles within the endosome, leading to establishment of a multi-vesicular body (MVB) [57]. Subsequently, the MVB binds to the plasma membrane in a calcium-dependent manner, is secreted into the environment, and releases its content in the form of ‘exosomes’ [58]. The formation of exosomes is a highly regulated process, and some of the key steps that control exosomal biogenesis have now been elucidated. One of the most critical proteins, ALIX, regulates the process of invagination in the endosomes, and subsequently enhances the production of exosomes [59,60]. In addition, heparanase has been identified to control intra-luminal budding of endosomes [61,62]. Once exosomes are released into the environment, they can be adsorbed by the recipient cells through several well-characterized mechanisms including: fusion with the plasma membrane, internalization through endocytosis, and binding to cell surface receptors [63].

One of the critical mechanisms by which host cells control the function of exosomes is through packaging of their cellular content. Although endosomal-sorting complex required for transport (ESCRT) primarily plays a key functional role in MVB transportation, this pathway has also been identified to facilitate packaging of exosomes [64]. The ESCRT complex comprises of five major proteins ESCRT-0, -I, -II, and -III, and the ATPase Vacuolar protein sorting-associated protein 4A (VPS4) that directs the packaging of proteins within the exosomes through process of recognition and sorting of ubiquitinated-modified cargo [65–70]. Furthermore, ESCRT-complex associated proteins, including TSG101 and VPS23, have also been shown to regulate exosomal packaging and recycling [65]. In contrast to ESCRT-mediated packaging of exosomes, several ESCRT-independent mechanisms have also been identified. One such process is regulated by ceramide, a trigger for budding and formation of ILVs within MVBs [71–73]. Interestingly, ceramide formation is catalyzed by neutral sphingomyelinase-2 (nSMase-2) and inhibition of this protein has been shown to inhibit exosomal production [71]. While exosomes are generated through a complex, tightly regulated process that require further interrogation, it is now well-recognized that these small vesicles do have very specific and important biological roles.

Potential of Exosomes as Cancer Biomarkers

It is becoming apparent that the Achilles' heel of the current generation of blood-based cancer biomarkers is their lack of sensitivity and/or specificity, prohibiting their widespread and routine use in the clinic. This is not surprising considering that the current approaches are aimed at detecting a miniscule signal produced by a relatively small number of cancer cells in the background of large body of signal produced by the normal cells. However, since the recognition that exosomes have tissue and/or organ-specificity combined with their easy detection in serum, it is becoming increasingly attractive to exploit their potential as cancer biomarkers. Fortunately, another school of thought favoring this rationale is that cancer cells appear to secrete larger quantities of exosomes vis-à-vis normal cells [74,75]. Although the underlying mechanisms for this disparate secretion of exosomes in cancer vs. normal cells remains unclear, it is believed that the low pH associated with the tumor microenvironment favors production of exosomes in cancer cells and their subsequent uptake or fusion by the target cells [76,77]. Another advantage of using cancer exosomes as substrates for biomarker development is that these vesicles are relatively stable, even at room temperature, for several weeks [78]. This eliminates the concern for confounding factors such as variability associated with clinical specimen collection, storage, and downstream analytical approaches [78,79]

Cancer-derived exosomes enhance tumor progression and metastasis by regulating various cancer-related pathways that control angiogenesis [80–88], modulation of stromal cells [89–91], remodeling of the extracellular matrix [84,92], and establishment of pre-metastatic niche [93–97]. A recent rodent study demonstrated that exosomes derived from specific cancers (brain, lung and liver) can initiate the generation of the pre-metastatic niche at a distinct organ site [98]. This study also demonstrated that exosomal protein markers could be used as a predictive marker for organ specific metastasis. Furthermore, breast cancer-derived extracellular vesicles were shown to preferentially promote breakdown of the blood-brain barrier and facilitate brain metastasis [99]. In contrast, exosomes isolated from bone

barrow mesenchymal stem cells altered cellular phenotype and promoted breast cancer cell dormancy [100]. In Figure-2, we provide an illustrated summary of how exosomes regulate transfer of oncogenic factors from malignant cells to target cells and how they drive cancer progression and metastasis. Interestingly, cancer exosomes are also involved in the regulation of the immune system [101]. Cancer cells release exosomes to evade the body's own innate immune responses, thereby providing protection from lysis by the complement-mediated membrane attack complexes [102,103].

One of the clever strategies for utilizing exosomes as cancer biomarkers is to target antigens present on their surface membranes to selectively identify organ and/or cellular origin [50]. In this regard, several organ-specific antigens on exosomes have already been reported in the literature [11,104,105]. Among the most promising candidates, prostate membrane specific antigen (PMSA) which is used to detect prostate-specific exosomes, was reported to be elevated in the blood of prostate cancer patients [105]. Furthermore, CD20, a marker of B-cell differentiation, was noted exclusively on B cell-derived exosomes [106]. Recent discovery of glypican-1 (GPC1), a GPI-anchored heparin sulfate proteoglycan, which could be used to identify cancer-specific exosomes has provided a compelling proof-of-concept for their potential use in accurately diagnosing cancer patients [11]. In this study, exosomal GPC1 was enriched specifically in cancer exosomes, and distinguished patients with pancreatic ductal adenocarcinoma from healthy volunteers and patients with benign pancreatic conditions with a perfect sensitivity and specificity of 100%. Functionally, GPC1 binds to VEGF and acts as an extracellular chaperone that restores its receptor binding affinity [107–109]. In addition, GPC1 appears to regulate the oncogenic Wnt signaling pathway [110,111]. Although follow-up, large-scale clinical trials are required to confirm the efficacy of GPC1 as a diagnostic marker for pancreatic cancer, this finding has been a seminal step towards establishing exosomal proteins as bona fide non-invasive cancer diagnostic biomarkers. Furthermore, the new found prominence of exosomal research has reignited the investigation of well-studied blood-based protein cancer markers such as EpCAM and CEA. Interestingly, both proteins were found to be highly over-expressed on the surface of exosomes derived from colorectal adenomas and cancers [49,112]. Similarly, proteins typically upregulated in ovarian (CA125) and breast (Her2/Neu) cancers were found in exosomes from ovarian cancer patients, and could be targeted as potential cancer biomarkers [113]. Based on the characterization of exosomes derived from ovarian cancer cell lines, CA125 as well as CD24, MUC-18, and EGFR have also been proposed to be potential candidates for detection of ovarian cancer [114]. Likewise, MUC-1, which was preferentially expressed on exosomes derived from breast cancer cell lines may facilitate detection of this malignancy [115].

Exosomal Markers in Colorectal Cancer

In contrast to several other human cancers, exosomal markers in CRC are not as thoroughly investigated at this time. Nonetheless, few studies have sequenced exosomes derived from patients with CRC [49,116]. Based upon the evidence thus far, CRC patients may display a number of exosomal protein markers including: vaccinia virus antigen A33, epithelial cell adhesion molecule (EpCAM), cadherin-17, carcinoembryonic antigen (CEA), proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), mucin 13 (Muc13),

misshapen-like kinase 1 (MINK1), keratin 18, mitogen-activated protein kinase 4 (MAPK4), claudin-1, -3, and 7, and ephrin-B1 and -B2 [50]. Based upon their expression pattern, CRC exosomes are thought to be divided into two major groups – one with protein characteristics of the basolateral regions (A33+), and the other with the apical regions (EpCAM+) within the colonic epithelium [49]. Furthermore, these two types of exosomes are also enriched with major histocompatibility complex class molecule II (MHC II), CD26, and CD63 proteins [50,117]. Functionally, these intestinal epithelial exosomes preferentially displaying MHC II complexes interact with dendritic cells and facilitate immune surveillance at the mucosal surface [118].

Evaluation of the protein and miRNA content in exosomes produced by CRC cell lines and normal colorectal epithelium revealed that exosomes derived from CRC cell lines possessed high levels of putative oncogenic miRNAs that were linked to metastasis and tumor progression [49]. Similarly, another study in which exosomes were isolated from the isogenic pairs of primary and metastatic CRC cell lines, SW480 and SW620 respectively, revealed that in contrast to SW480, exosomes from SW620 cells were enriched with metastatic factors including MET and TNC [84]. These findings are in line with data from other cancers and clearly demonstrate that CRC-derived exosomes reflect the origin of their respective cell/organ types. Recently, a group from Japan established a highly sensitive analytical technique for the profiling of surface proteins in extracellular vesicles called “ExoScreen” [119]. Using CD147, a surface antigen identified in CRC cell lines, they demonstrated that extracellular vesicular CD147 could out-perform CEA and CA19-9 as a CRC diagnostic marker. Another potentially important feature of CRC-exosomes is that they express high levels of GPC1, a putative cancer exosomal marker discovered in pancreatic cancer cells [11]. Interestingly, characterization of low molecular weight proteins (<3kDa) secreted from the two CRC cell lines identified GPC1 as one of most abundant GPI-anchored proteins secreted by CRC cells [120,121]. Thus, use of cancer-related proteins or RNA markers in conjunction with the exosomal levels could significantly improve the ability of serum-based CRC biomarkers.

Exosomal miRNAs

MiRNAs are small non-coding single-stranded RNAs, approximately 19–24 nucleotides in length, known to post-transcriptionally regulate gene-expression by binding to the 3'-untranslated regions (UTRs) of target mRNAs. This process leads to gene silencing through degradation, destabilization of the mRNA cap and tail, or by steric blocking of the ribosome binding complex [122]. MiRNAs play a central role in various physiological processes, are frequently dysregulated in human cancers, and are currently being explored for their potential as free circulating biomarkers [123]. Unfortunately, till date, independent studies have failed to develop clinically-actionable miRNA biomarkers due to a variety of reasons including the variability in their expression depending upon the methods used to process clinical specimens, analytical approaches used and the strategies employed for data normalization [124]. Previous studies have indicated that the majority of miRNA signal in serum is primarily contributed by free-floating RNAs [125,126], while a recent study discovered that miRNA expression in serum and saliva was predominantly derived from exosomes [127]. Although more studies are needed to resolve this issue, it is becoming

apparent that while the utility of circulating miRNAs as cancer biomarkers is promising, targeting exosomal miRNA signatures in blood may be more prudent. Nevertheless, many studies have now consistently demonstrated that exosomal miRNAs influence both the physiology of the recipient cells and regulate oncogenic properties in cancer. Figure 3 depicts how miRNAs are packaged into exosomes and systematically target recipient cells.

Intriguingly, a recent study discovered the underlying mechanisms for the observed discrepancy in miRNA content between cancer and normal exosomes [128]. This study showed that exosomes from breast cancer cells contain the RISC-loading complex which is responsible for converting pre-miRNAs to mature-miRNAs, thus enriching miRNAs within cancer exosomes – a feature that is absent in normal breast cells. Furthermore in another study, researchers demonstrated that expression of VPS4A, a protein involved in exosomal packaging, regulated exosomal miRNA levels in hepatocellular carcinoma (HCC) [129]. Overexpression of VPS4A enhanced tumor-suppressive miRNA content in exosomes, while overall production of exosomes was attenuated in HCC cell lines. Other experiments confirmed that exosomal miRNA packaging is driven by miRNA characteristics of the parental cells in breast [128], glioblastoma [83], and ovarian [74] cancers, as well as in T- and B-cells [130]. Collectively, these data provide important insights into how exosomal miRNAs are packaged, and hence, how these unique features can be exploited for their development as cancer biomarkers.

As targets of exosomal cancer biomarkers, both miRNAs and proteins appear to have distinct advantages. While proteins on the surface of exosomes can be measured using antibodies, remarkable stability of miRNAs makes them more attractive for older retrospectively collected samples [123]. Furthermore, the recent discovery of RISC-loading complex in cancer exosomes suggests substantially higher miRNA expression difference between cancer and normal exosomes [128].

MiRNAs Expression profiles in Cancer Exosomes

Cell Lines: While characterization of exosomal miRNAs from clinical specimens are still limited, a large body of data for exosomal miRNA profiles from various types of cancer cell lines has now been generated (Table 1). In breast cancer, microarrays were used to profile exosomal miRNAs derived from metastatic MDA-MB-231 and non-metastatic MCF-7 cell lines [131]. These studies revealed upregulation of miR-130a and miR-328 in exosomes derived from metastatic MDA-MB-231 cells, while MCF-7-derived exosomes exhibited higher expression of miR-301a, miR-34a, and miR-106b. Likewise, deep sequencing was performed on exosomes derived from MDA-MB-231 cells and non-malignant MCF-10 breast cancer cells [80], leading to the identification of miR-105 as the most upregulated exosomal miRNA, which was responsible for promoting metastasis by suppression of the tight junction protein ZO-1. In another study on prostate cancer, exosomal miRNA profiling in PC-3 cancer cells and RWPE-1 normal prostate epithelial cells revealed a panel of 80 miRNAs that were specifically expressed in PC-3 vs. RWPE-1-derived exosomes [132].

Selective packaging of exosomal miRNAs was identified in gliomas. A comparison of non-coding RNA composition between cancer exosomes and parental cell lines showed enrichment of several functionally uncharacterized miRNAs in cancer exosomes, including

miR-451, miR-4792, miR-1273, miR-1269b, miR-4500, miR-1273d, miR-4443, and miR-1273a [133]. Similarly, in HCC, an array-based comparison between cancer exosomes and parental cells identified 55 differentially expressed miRNAs with at least four-fold change in expression in cancer exosomes [134]. Interestingly, 11 of these miRNAs were detected exclusively in exosomes. Collectively, characterization of exosomes in cultured cell lines has provided a blue print to narrow down potential target exosomal miRNAs that can subsequently be evaluated in clinical samples for their biomarker potential.

Clinical Studies: Although this is an evolving field, several clinical studies have already evaluated the efficacy of exosomal miRNAs as cancer biomarkers in patient specimens, as summarized in Table 2. In prostate cancer, a microarray-based approach was used to analyze miRNA profiles in plasma-derived exosomes from 78 metastatic prostate cancer patients vs. 28 healthy controls. A total of 11 miRNAs were upregulated (miR-107, miR-130b, miR-141, miR-181a-2, miR-2110, miR-301a, miR-326, miR-331-3p, miR-432, miR-484, miR-574-3p, miR-625) and one downregulated (miR-181a-2) in exosomes from metastatic prostate cancer patients compared to healthy controls [135]. This study thereafter analyzed plasma exosomes from 55 non-metastatic and 16 metastatic prostate cancer patients and identified 15 miRNAs were upregulated (miR-582-3p, miR20a, miR-375, miR-200b, miR-379, miR-513-5p, miR-577, miR-23a, miR-1236, miR-609, miR-17, miR-619, miR-624, miR-198 and miR-130b) and one was downregulated (miR-572) in metastatic vs. non-metastatic prostate cancer exosomes. Another study assessed four well-studied pancreatic cancer-associated oncogenic miRNAs (miR-17, miR-21, miR-155 and miR-196a) in exosomes from 49 pancreatic adenocarcinoma patients and demonstrated an elevation of miR-17 and miR-21 compared to healthy controls [136]. In HCC, microarray-based profiling initially identified exosomal miR-718 as the most differentially expressed, and high levels of this miRNA were subsequently found to be associated with tumor aggressiveness and recurrence [137].

In a biomarker study for ovarian cancer, expression patterns of eight miRNAs that were previously found to be important in diagnosing ovarian cancer, were evaluated in exosomes [74]. Expression of these non-coding RNAs were compared in exosomes, tumor tissues and sera from fifty women diagnosed with serous papillary adenocarcinoma of the ovary (Stage I-IV), ten women with benign ovarian adenomas, and ten healthy females. Of interest, the expression profiles of these eight miRNAs were similar in tumor cells and serum exosomes from patients with ovarian cancer, but differed significantly when compared to patients with benign disease. Remarkably, exosomal miRNAs were not detected in healthy subjects, highlighting the tumor-specificity of exosomal miRNAs as disease biomarkers.

While evaluating the role of exosomal miRNAs in esophageal cancer, 51 patients with esophageal squamous cell carcinoma (ESCC) were compared with 41 control individuals, to identify exosomal miR-21 expression to be significantly elevated in ESCC samples [138]. In breast cancer, exosomal miR-373 expression was significantly upregulated in triple-negative (negative for the estrogen receptor, progesterone receptor, and Her2/Neu receptor) breast cancers [139]. Collectively, the results of these studies highlight the significant body of data demonstrating progress made in the identification of exosomal miRNA-based cancer biomarkers – a concept that is very likely going to gain further traction in the coming years.

Exosomal miRNAs in Colorectal Cancer

In CRC, several miRNAs have been identified as potential serum- and tissue-based cancer biomarkers [140]. In particular, miR-21, appears to be one of the most promising serum-based miRNAs for the early detection of disease [19], which has now also shown to be highly expressed in exosomes [138]. Serum miR-21 is frequently upregulated in CRC patients in a stage-dependent manner [19]. However, despite its robustness as a potential non-invasive biomarker, miR-21 expression is not specific for CRC, as it is frequently upregulated in other cancers including: breast [141], ovarian [142], cervical [143], pulmonary [144], esophageal [145], cephalic [146], and hepatocellular [147] cancer. Its expression is also elevated during inflammation [148]. This is a common concern with many other miRNA biomarkers, as they are simultaneously dysregulated in multiple cancers [149]. Considering that miRNA profiles of CRC-derived exosomes were evaluated in both clinical and cultured cells, it is possible that CRC-specific exosomal miRNAs will be eventually identified.

A recent study profiled exosomal miRNAs derived from CRC patients and compared these with healthy controls, and identified 16 differentially expressed miRNAs including: let-7a, miR-21, miR-23a, miR-150, miR-181b, miR-181d, miR-223, miR-483-5p, miR-638, miR-1224, miR-1229, miR-1246, miR-1268, miR-1290, miR-1308, and miR-1915 [150]. Subsequent analysis revealed miR-1246 and miR-23a, had the highest sensitivity of 95% and 92% respectively, and appear to be promising diagnostic markers for CRC. Another study interrogated the use of CRC-exosomal miRNAs as biomarkers for cancer recurrence and identified differential expression of the miR-17-92 cluster following microarray analysis. The expression of exosomal miR-19a, specifically, was highly upregulated in both early and advanced stages of CRC and correlated with key prognostic indices [151]. In addition, metastasis associated miRNAs including miR-21, miR-192 and miR-221 were detected from exosomes isolated from three CRC cell lines (HCT-15, SW480, and WiDr) using an exosomal marker CD81 [97].

Two major colorectal exosomal and microvesicular populations (A33 and EpCAM-positive) derived from CRC cell lines were comprehensively characterized by next generation sequencing [116]. Interestingly, the miRNA expression profiles of each vesicle were distinct, and certain miRNAs were found to be selectively packaged in exosomes in specific cell lines. Furthermore, a recent study using a series of *in vitro* experiments demonstrated that drug resistant cell lines secrete exosomes with low levels of the tumor-suppressive miR-200 family members compared to their isogenic parental counterparts [152]. Interestingly, cells that demonstrated loss of expression for miR-200 family members in exosomes were more likely to invade the blood or lymphatic system. In a comparison between exosomes isolated from *KRAS* mutant and wild type isogenic CRC cell lines, distinct differences were observed in their miRNA profiles. In particular, miR-10b was selectively upregulated in wild type exosomes, while miR-100 was upregulated in *KRAS* mutant exosomes, indicating possible *KRAS*-dependent miRNA export to exosomes in CRC [153].

Conclusions

Accumulating evidence suggests that exosomes released from cancer cells have a major role in promoting oncogenesis. These vesicles inherit characteristics of the parental cancer cells including their protein, mRNA, and miRNA profiles, which eventually affect the tumorigenic potential of cancer exosomes. Molecular characterization of exosomes will not only unravel their functions, but will also help in developing robust blood-based noninvasive cancer-specific detection approaches. Accordingly, next-generation sequencing of CRC tissues has allowed us to comprehensively analyze the miRNA profile of CRC [154,155]. Similarly, several studies have already profiled exosomal miRNAs in CRC clinical specimens and in cell lines [116,150]. Comparison between the profiling of tumor samples and exosomes provides unique insight into which miRNAs are packaged into cancer exosomes from the tumor and may results in the identification of a novel diagnostic marker. Over the last decade, miRNAs have emerged as one of the most attractive blood-based cancer biomarker candidates primarily due to two reasons – their elevated expression in cancer, and their high abundance and remarkable stability in blood. Although assessment of miRNA levels in serum has yielded significant promise for the development of potential diagnostic and prognostic biomarkers in CRC, it is quite plausible that quantifying their expression in exosomes will add another layer of specificity in improving their clinical significance as cancer biomarkers. Advancements in this research field will undoubtedly contribute towards the discovery of new diagnostic and prognostic CRC biomarkers, and some of these molecular signatures may eventually be translated for the development of therapeutic options in CRC patients. In essence, we may just have yet only seen the tip of the iceberg – excitement is building, and next few years will unravel the mystery around this topic and will likely yield additional evidence for exosomal miRNA biomarkers as the emerging frontiers in CRC and other human cancers.

Expert Commentary and Five-Year View

Colorectal cancer is a highly curable disease when detected early, and regular screening within the high-risk population has been recognized as a key strategy for reducing the incidence of this fatal disease. In this context, availability of highly sensitive, non-invasive blood-based assays for the detection of advanced adenomas and early-stage colorectal cancers may result in significant attenuation in the mortality associated with this cancer. Currently, there is a great deal of optimism for the development of methylated DNA and exosomal miRNA biomarkers for the early detection of colorectal neoplasia.

Strategically, there are two logical approaches to further improving the detection rates of these epigenetic biomarkers in the blood of CRC patients. The first strategy involves discovery of a novel epigenetic marker which can outperform the current generation of best-performing biomarkers. Without a doubt, with the availability of next generation sequencing platforms, such a discovery approach will generate a vast array of novel biomarkers over the next five years. However, the real challenge will be to sift through thousands of probable markers, identify the most promising ones, and perform validation of the select few in large-scale independent patient cohorts. To date, some of the major issues associated with miRNA markers have been twofold: first, their low levels of expression in blood, making it difficult to accurately quantify their expression using conventional techniques. The second issue has

been their lack of specificity for accurately identifying their origin from the disease site. For the former issue, newer quantification methodologies such as digital PCR with superior detection thresholds may improve the quantification of low expression level miRNAs in serum. However, improving the specificity of these circulating miRNA biomarkers still presents a significant challenge. In order to overcome these challenges, one possibility would be to investigate their expression in exosomes released from cancer cells, which will allow for easier and possibly more specific identification of these biomarkers noninvasively in blood. Since exosomes inherit characteristics of their parental cells, it is possible to target specific exosomal fractions that are derived specifically from the colon, and other human cancers.

Finally, it is important to remind ourselves that even though colonoscopy is an accurate screening protocol, it may be impractical to reduce its invasive nature, the expense associated with this procedure, and lower compliance rates associated with CRC screening among general population. In other words, one must not forget that there is a delicate balance between assay accuracy, cost, and convenience of the screening protocol. The most clinically relevant CRC biomarkers should score high on all three fronts, which will improve our understanding and will permit technological advancements for developing superior screening methodologies for CRC in the future. We are currently witnessing the beginning of a new era in the cancer biomarker field and hopefully breakthroughs will arrive sooner than our anticipation.

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* *Of interest*

** *Of considerable interest*

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Key issues

- A blood-based non-invasive colorectal cancer early detection biomarker is needed to reduce mortality associated with the disease
- Exosomes are phospholipid bilayer nano-vesicles, 30–140 nm in diameter, excreted by a wide variety of cells and found in various bodily fluids
- Exosomes inherit molecular constituents of their cell of origin, and this property could be used to detect exosomal signals released from cancer cells
- Evaluation of exosomal miRNAs should significantly improve detection rates of conventional serum based cancer biomarkers
- Cancer can process pre-miRNAs to mature miRNAs within exosomes, which subsequently results in differential expression profiles between normal and cancer cell-derived exosomes
- Certain miRNAs appear to exist exclusively in cancer exosomes, but not cancer cells, which provides a unique opportunity for developing them as disease-specific biomarkers

Figure 1. Biogenesis and composition of exosomes

In the host cell, the phospholipid bilayer of the early endosome invaginates to form multiple intra-luminal vesicles (ILV) within the lumen; such endosomal complex containing ILVs is termed multi-vesicular body (MVB). MVBs subsequently binds to the plasma membrane in a calcium-dependent manner and releases the ILVs into the extracellular environment. ILVs are now called an “exosomes”. Exosomes released into the environment can be absorbed by recipient target cells via multiple mechanisms including: fusion with the plasma membrane, internalization through endocytosis, and binding to cell surface receptors. Currently, exosomes are primarily classified by their size range of 30–140 nm. Exosomes contain several surface markers such as annexins, Rabs, ALIX, TSG101, CD9, CD81, and CD63. Exosomes can also carry oncogenic surface proteins characteristic of the host cancer cell such as EGFR or EpCAM.

Figure 2. Oncogenic functionality of tumor-derived cancer exosomes

Cancer cells secrete exosomes containing oncogenic characteristics of the host cancer cells into the environment. The cancer-derived exosomes can bind to several target cells and exert various oncogenic effects including: binding to cancer cells and accelerating cancer progression and metastatic potential of other cancer cells (upper panel, blue arrows), binding to endothelial cells within blood capillaries (in red) and inducing gap-junction breakage causing vascular leakage, or binding to distant normal cells and initiating metastatic niche formation (green arrows). The metastatic tumor cells later enter the circulation via the endothelial gaps in blood vessels and travel to the distant organ pro-metastatic niche.

Figure 3. Generation and uptake of cancer-derived exosomal miRNAs

Cancer cells transcribe pre-miRNAs in the nucleus. The pre-miRNAs are thereafter transported from the nucleus into the cytoplasm. miRNAs are engulfed into a multivesicular body (MVB) as both pre-miRNAs or mature-miRNAs. The packaged ILVs are then released into the cell environment via fusion of the MVB to the cell plasma membrane. The exosome travel to the target cell and are taken up by various mechanisms. Subsequently, the packaged pre-miRNAs and mature miRNAs are released into the target cell cytoplasm. The mature miRNAs bind to its homologous mRNAs within the 3' UTR to initiate degradation. This process results in down regulation of specific target genes and thereby alters gene expression.

Table 1

Exosomal miRNA expression profiles in cancer cell lines

Tissue	Cell-Type	miRNA	Levels	Comparison	Function	Key target genes	Ref
Breast Cancer	MCF-7	16-5p	Detected	Intra vs. Extra cell	Tumor suppressor	BCL2	[125]
		21-5p	Detected	Intra vs. Extra cell	Oncogene	PTEN	[125]
		34a-5p	↑	MDA-MB-231	Tumor suppressor	SIRT1	[131]
		106b-5p	↑	MDA-MB-231	Oncogene	BRMS1	[131]
		210-3p	↑	MCF-10	Angiogenesis		[72]
		221-3p	↓	MCF-7 (Tamox)	Tamoxifen resistance		[129]
	MCF-10DCIS Stem cells	222-3p	↓	MCF-7 (Tamox)	Tamoxifen resistance		[129]
		140-5p	↓	MCD-10DCIS	Regulate Stemness	SOX9	[156]
		Let-7a-5p	↑	MCF-7	Tumor suppressor	RAS	[131]
		10b-5p	↑	MFC-10a	Oncogene	HOXD10	[157]
Ovarian	4T1	16-5p	Detected	Intra vs. Extra cell	Tumor suppressor	BCL2	[125]
		21-5p	Detected	Intra vs. Extra cell	Oncogene	PTEN	[125]
		24-3p	Detected	Intra vs. Extra cell	Tumor suppressor	BCL2, RegIV	[125]
		210-3p	↑	MCF-10	Angiogenesis		[72]
		9-5p	Detected	Cel-miR-39	Angiogenesis	JAK-STAT	[158]
		96-5p	Detected	Cel-miR-39	Oncogene	FOXO3a	[158]
	SKOV-3	210-3p	↑	MCF-10	Angiogenesis		[72]
		Let-7a-f-5p	↑	OVCAR-3	Tumor suppressor	RAS	[159]
		200a-c-3p	↑	SKOV-3	Tumor suppressor		[159]
		10a-5p	↓	RWPE-1	Oncogene	HoxD10	[132]
Prostate	PC-3M	16-5p	↓	PNT-2	Tumor suppressor	BCL2	[160]
		34a-5p	↑	PC-3RD	Tumor suppressor	BCL2	[161]
		143-3p	↓	PNT-2	Tumor suppression	K-RAS	[160]
		205-5p	↓	PNT-2	Oncogene	Zeb1/2	[160]
	DU145	34a-5p	↑	DUI145RD	Tumor suppressor	BCL2	[161]

Tissue	Cell-Type	miRNA	Levels	Comparison	Function	Key target genes	Ref
		195-5p	↑	RWPE-1	Tumor suppressor	STIM1	[132]
		221-3p	↑	RWPE-1	Oncogene	RB1	[132]
	22Rv1	34a-5p	↑	22Rv1RD	Tumor suppressor	BCL2	[161]
Kidney		16-5p	↓	Parental cell	Tumor suppressor	BCL2	[125]
	HEK293	21-5p	↓	Parental cell	Oncogene	PTEN	[125,162]
		24-3p	Detected	Cel-miR-39	Tumor suppressor	BCL2, RegIV	[125]
Glioma	U251	21-5p	↑	Parental cell	Oncogene	PTEN	[133]
		1246-5p	↑	Parental cell	Oncogene	P53	[133]
Bladder	T24	23b-3p	↑	Parental cell	Tumor suppressor		[156]
	FL3	23b-3p	↑	Parental cell	Tumor suppressor		[156]
		142-5p	↑	Parental cell	Oncogene	FLOT1	[134]
		367-3p	↑	Parental cell	Oncogene	RYR3	[134]
	Hep3B	376a-3p	↑	Parental cell	Tumor suppressor	PIK3R,	[134]
		378a-3p	↑	Parental cell	Oncogene	Sufu and Fus-1	[134]
Hepatoma	SMMC- Vsp4a+	92a-3p	↑	SMMC (wt)	Oncogene	BIM	[129]
		34a-5p	↑	SMMC (wt)	Tumor suppressor	BCL2	[129]
		122-5p	↑	SMMC (wt)	Tumor suppressor	ADAM17	[129]
		10a-5p	↑	Parental cell	Oncogene	HoxD10	[129]
		10b-5p	↑	Parental cell	Oncogene	MICB	[129]
		21-5p	↑	Parental cell	Oncogene	PTEN	[129]
	SMMC- 7721	92a-3p	↑	Parental cell	Oncogene	BIM	[129]
		191-5p	↑	Parental cell	Oncogene	SATb1	[129]
ColorectalCancer		Let-7a-5p	↑	FHC	Tumor suppressor	RAS	[150]
	HCT116	21-5p	↑	FHC	Oncogene	PTEN	[150]
		1246-5p	↑	FHC	Oncogene	P53	[150]
	SW48	Let-7a-5p	↑	FHC	Tumor suppressor	RAS	[150]
		21-5p	↑	FHC	Oncogene	PTEN	[150]

Tissue	Cell-Type	miRNA	Levels	Comparison	Function	Key target genes	Ref
		1246-5p	↑	FHC	Oncogene	P53	[150]
SW480		21-5p	↑	U6 snRNA	Oncogene	PTEN	[97]
		92a-3p	↑	Tissue	Oncogene	Dkk-3	[163]
		1246-5p	↑	Parental Cell	Oncogene	PML	[164]
SW620		200a-5p	↑	SW620-RH	Tumor Suppressor	ZEB	[152]
WiDR		21-5p	Detected		Oncogenic	PTEN	[97]
		34a-5p	Detected		Tumor suppressor	SIRT1	[97]
		92a-3p	↑	Tissue	Oncogenic	Dkk-3	[163]
		1246-5p	↑	Parental cell	Oncogenic	PML	[164]
HT29		Let-7a-5p	↑	FHC	Tumor suppressor	RAS	[150]
		21-5p	↑	FHC	Oncogenic	PTEN	[150]
		1246-5p	↑	FHC	Oncogenic	P53	[150]
RKO		Let-7a-5p	↑	FHC	Tumor suppressor	RAS	[150]
		21-5p	↑	FHC	Oncogenic	PTEN	[150]
		1246-5p	↑	FHC	Oncogenic	P53	[150]
COLO201		92a-3p	↑	Tissue	Oncogenic	Dkk-3	[163]
		1246-5p	↑	Parental cell	Oncogenic	PML	[164]
DLD-1		92a-3p	↑	Tissue	Oncogene	BIM	[163]
		100-5p	↑	Dks-8	Oncogene	E-Cadherin	[153]
		1246-5p	↑	Parental cell	Oncogene	PML	[164]
DKO-1		100-5p	↑	Dks-8	Oncogene	E-Cadherin	[153]
LIM1863		19a/b-3p	↑	Parental cell	Oncogene	PTEN	[116]
		106a-5p	↑	Parental cell			[116]
		378a-3p	↑	Parental cell			[116]
		423-3p	↑	Parental cell			[116]
		532-3p	↑	Parental cell			[116]
		577-5p	↑	Parental cell			[116]

Table 2

Exosomal miRNA expression profiles in human cancers

Tumor type	Sample type	miRNA	Levels	Function	Key target genes	Ref.
Prostate	Serum	301a-3p	↑	Oncogene	PTEN	[135]
	Serum	375-3p	↑	Tumor suppressor	PKD1	[135,165]
	Serum	432-5p	↑	Oncogene	ADAR1	[135]
Pancreatic adeno-carcinoma	Serum	17-5p	↑	Oncogene	PI30	[136]
	Serum	21-5p	↑	Oncogene	PTEN	[136]
Esophageal squamous cell carcinoma	Serum	21-5p	↑	Oncogene	PTEN	[138]
Breast cancer	Ascites/Serum	21-5p	↑	Oncogene	PTEN	[166,167]
	Serum	101-3p	↑	Oncogene	Janus Kinase 2	[139]
	Ascites	205-5p	↑	Tumor suppressor	Her3	[166]
	Serum	373-3p	↑	Oncogene	Camptothecin	[139]
Ovarian cancer	Ascites/Serum	21-5p	↑	Oncogene	PTEN	[74,166,168]
	Serum	23b-3p	↑	Tumor suppressor	RUNX2	[168]
	Serum	29a-3p	↑	Tumor suppressor	p42.3	[168]
	Serum	99a-5p	↑	Oncogene	FGFR3	[168]
	Serum	125b-5p	↑	Tumor suppressor	ARID3B	[168]
	Ascites/Serum	205-5p	↑	Tumor suppressor	Her3	[74,166]
Hepato- cellular carcinoma	Serum	214-3p	↑	Oncogene	P53	[74]
	Serum	718-3p	↓		HOXB8	[137]
Lung cancer	Plasma	Let-7f-5p	↑	Tumor suppressor	RAS	[169]
	Plasma	17-3p	↑			[170]
	Ascites/Plasma	21-5p	↑	Oncogene	PTEN	[170]
	Plasma	106a-5p	↑	Oncogene	TIMP2	[170]
	Plasma	192-5p	↑	Tumor suppressor	BCL2	[170]
	Ascites/Plasma	205-5p	↑	Tumor suppressor	Her3	[170]
	Plasma	210-3p	↑	Oncogene	VMP1	[170]

Tumor type	Sample type	miRNA	Levels	Function	Key target genes	Ref.
Glioblastoma	Plasma	212-3p	↑	Tumor suppressor	MeCP2	[170]
	Plasma	223-3p	↑	Tumor suppressor	STMN1	[169]
	Serum	Let-7a-5p	↑	Tumor suppressor	RAS	[83]
	Serum	15b-5p	↑	Tumor suppressor	Cyclin D1	[83]
	Serum	16-5p	↑	Tumor suppressor	BCL2	[83]
	Serum	19b-5p	↑	Oncogene	TP53	[83]
	Serum	21-5p	↑	Oncogene	PTEN	[83]
	Serum	320	↑	Tumor suppressor	TFR-1	[83]
	Serum	Let-7a-5p	↑	Tumor suppressor	RAS	[150]
	Serum	19a-3p	↑	Oncogene		[151]
Colorectal cancer	Serum	21-5p	↑	Oncogene	PTEN	[150]
	Serum	92a-3p	↑	Oncogene	DKK-3	[163]
	Serum	181d-5p	↑	Tumor suppressor	KRAS and BCL2	[150]
	Serum	483-5p	↑	Oncogene	RHoGDI1	[150]
	Serum	1224-5p	↑	Oncogene	CREB1	[150]
	Serum	1246-5p	↑	Oncogene	P53	[150]
	Serum	Let-7a-5p	↑	Tumor suppressor	RAS	[150]
	Serum	19a-3p	↑	Oncogene		[151]
	Serum	21-5p	↑	Oncogene	PTEN	[150]
	Serum	92a-3p	↑	Oncogene	DKK-3	[163]