Small extracellular vesicle DNA-mediated horizontal gene transfer as a driving force for tumor evolution: Facts and riddles

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The basis of the conventional gene-centric view on tumor evolution is that vertically inherited mutations largely define the properties of tumor cells. In recent years, however, accumulating evidence shows that both the tumor cells and their microenvironment may acquire external, non-vertically inherited genetic properties via horizontal gene transfer (HGT), particularly through small extracellular vesicles (sEVs). Many phases of sEV-mediated HGT have been described, such as DNA packaging into small vesicles, their release, uptake by recipient cells, and incorporation of sEV-DNA into the recipient genome to modify the phenotype and properties of cells. Recent techniques in sEV separation, genome sequencing and editing, as well as the identification of new secretion mechanisms, shed light on a number of additional details of this phenomenon. Here, we discuss the key features of this form of gene transfer and make an attempt to draw relevant conclusions on the contribution of HGT to tumor evolution.

KEYWORDS
extracellular vesicles, exosomes, horizontal gene transfer, tumor evolution, cell-cell communication
Introduction

Generally, it is considered that cancer cells divide by mitosis and do not exchange DNA with each other or with the cells of the tumor microenvironment (1, 2). The vertically inherited mutations across successive generations largely (but not exclusively) determine the adaptive reaction of the cell’s progeny against the existing selective pressure and result in the expansion or contraction of their subclones (1, 2). Recent studies suggested that a non-vertical transmission of DNA may also occur among the community of cancer cells (oncobiota) and between cancer- and microenvironmental genomes (3, 4). This process, namely horizontal gene transfer (HGT), may provide a selective advantage for the recipient cell if the overall effects of the transferred gene are beneficial (5). This phenomenon is considered as a non-cell-autonomous mechanism, in which higher genotype’s adaptive value of the recipient cell is partially linked to the donor cells. HGT may accelerate genomic evolution by allowing a faster adaptation in the group of recipient cells than it would happen by vertical transfer of the same gene (5). This exchange of genetic material occurs among both nearby cells (as paracrine signals) and relatively distant ones (in an endocrine manner) (3, 6). Biologically active cell-free DNA (cfDNA), as a mediator of HGT, can be transported in different forms in the intercellular space (Box 1). Here, we will discuss the main steps of the sEV-DNA-mediated HGT among donor and recipient cells in human cancers, and where it is possible, we compare it to the processes of normal mammalian cells. We particularly focus on the potential biological relevance of sEV-DNA and the controversial issues association with HGT in tumor evolution.

Release of DNA through sEV secretion

The majority of cancers are derived from a single ancestral cell by the generation of a diverse successor population with subclonal architecture (1). Cancer also shapes its own microenvironment into a supportive one (19). The developing genetic-, epigenetic-, functional- and phenotypic heterogeneity of individual cells provides a remarkable capacity for a population to adapt to challenging environmental conditions during cancer progression and therapy (20, 21). HGT can significantly influence the evolutionary trajectory of a given tumor by spreading genes encoding for molecules which provide advantages for the cells with suboptimal survival, expansion or metastatic capacity. EV-mediated HGT is not a common event among healthy mammalian cells (22). However, the fundamental differences between cancer and normal cells may change the frequency of the EV-mediated HGT. The first alteration in cancer can be the abnormal transport of the genomic DNA (gDNA) from the nucleus to the cytoplasm. Aberrant expression of nuclear membrane components, abnormalities in chromosome segregation, and mechanical forces from the actin cytoskeleton resulting in the rupture of the nuclear envelope are significantly more often observed in cancer than in normal cells, where the disintegration of the nuclear membrane is

**Box 1** Carriers of cfDNA, EV subcategories, sEVs, exosomes and non-conventionally released vesicles.

CfDNA as a mediator of HGT, can be present in different forms in the extracellular space, namely as DNA fragments, virionsomes [a complex of DNAs, RNAs, proteins, and lipids (7)], nucleosomes (DNA wrapped around an octamer of histone proteins), or packaged into extracellular vesicles (EVs) (8). The EV nomenclature refers to the EV biogenesis including: i) exosomes (sEVs with endosome/multivesicular body (MVB) origin; ~50-100 nm), ii) microvesicles (originated by direct budding/blebbing from the cell surface; 100-1000 nm) and iii) large EVs including apoptotic bodies (products of apoptotic cell disassembly; 1-5 µm) (9). In most instances, only the diameter of EVs can be determined with certainty. In this review, we follow the MISEV 2018 guidelines which suggest the term sEVs for EVs smaller than 200 nm in diameter, regardless of their origin (10). It follows from the above that the sEV term denotes a group of EVs with heterogeneous origins. Exosomes represent a subset of EVs which are formed intracellularly by the inward budding of the limiting membrane of endosomes/MVBs (with the intrusion of the cytosolic components). Later, the MVBs fuse with the plasma membrane, so their intraluminal vesicles are released into extracellular space as exosomes. After the uptake, exosomes are known to mediate a wide spectrum of effects on the recipient cell (11).

Recently, unconventional sEV release mechanisms have been hypothesized. In this case, migrasomes and en bloc released MVB-like EV clusters could possibly serve as sources of sEVs upon rupture of their limiting membrane (12, 13).
transient and limited to the mitosis (23). Disruption of the membrane barrier around the chromosomes allows gDNA to be exposed to cytoplasmic locations outside of the nucleus (Figure 1A) (23). Another form of delivery of gDNA to the cytoplasm may occur through micronucleus formation (Figure 1B), when a chromosome (or part of it) segregates improperly during mitosis. Disruption of the membrane barrier around the chromosomes allows gDNA to be exposed to cytoplasmic locations outside of the nucleus (Figure 1A). Another form of delivery of gDNA to the cytoplasm may occur through micronucleus formation (Figure 1B), when a chromosome (or part of it) segregates improperly during mitosis. Disruption of the exposed to cytoplasmic locations outside of the primary nuclear membrane (24, 25). Other, phenomena which are not studied in association with oncogenic sEV release, such as nucleophagy might also take a part in this process (26). Cytoplasmic or micronucleus-enclosed gDNA can translocate into the intraluminal vesicles of multivesicular bodies (MVBs) (27). exosome secretion may include receptor-mediated processes (characteristic of both normal and tumor cells, indicated by a red frame). In the cytoplasm, the sEVs (or their components) may activate DNA recognition pathways e.g., cGAS/STING. The dynamic adaptation of sEV-mediated DNA release is further supported by the changing quality and/or quantity of transported genetic content (e.g., the proportion of genomic and mitochondrial (mt) DNA, as well as the average size of DNA fragments) upon diverse environmental effects (29, 30).

Considering that the sEVs-conveyed gDNA fragments represent the entire host genome and contain full-length oncogene sequences (31), the sEVs may enter the nucleus through nuclear pores. The nuclear gDNA is discharged to the cytoplasmic region of the EV releasing cell (left) through the rupture of the nuclear envelope or by the formation of micronuclear (the fluorescent microscopic image shows a double-strand break (DSB) site in the micronucleus of HT-29 cell (white arrow, γH2AX staining, scale bar: 2 µm)] (B). (C) From the cytoplasm or from micronuclei the gDNA translocates into the intraluminal vesicles (future exosomes) of multivesicular bodies. (D) Both the gDNA content of the exosomes and their release are increased in tumor cells, especially upon the effect of therapeutic stress. (E) The gDNA may be transferred either in the lumen of sEVs and/or on the exofacial EV surface, or independently as a non-vesicular component. The gDNA content of sEVs might depend on their origin, like exocytosis of MVBs (D), amphisomes (F), or sEV discharge from en bloc released MVB-like EV clusters or migrasomes (G). The red frame indicates that all listed processes (i.e., migrasome formation, exosome secretion, amphisome exocytosis) may occur in both normal and tumor cells. (H) Uptake of the released sEVs by a recipient cell (right) may include receptor-mediated processes (characteristic of both normal and tumor cells, indicated by a red frame). (I) In the cytoplasm, the sEVs (or their components) may activate DNA recognition pathways e.g., cGAS/STING. (J) The EV-containing late endosomes may reach the nucleus. (K) Small vesicles (~200 nm) are also detectable in association with the nuclear membrane invaginations (red arrowheads in electron microscopy images of HT29 colorectal cancer cells). The origin of these vesicular structures has not been examined (scale bars: 500 nm). (L) The integration of gDNA into the recipient genome may require malfunction of the host DNA repair or onco-suppressor mechanisms (e.g., p53, and BRCA1). Many of the processes presented here have only been described in relation to cancers. Further studies are needed to demonstrate whether these occur in healthy cells.

FIGURE 1
A simplified representation of sEV-mediated HGT among tumor/microenvironmental cells. (A) The nuclear gDNA is discharged to the cytoplasmic region of the EV releasing cell (left) through the rupture of the nuclear envelope or by the formation of micronuclear (the fluorescent microscopic image shows a double-strand break (DSB) site in the micronucleus of HT-29 cell [white arrow, γH2AX staining, scale bar: 2 μm]) (B). (C) From the cytoplasm or from micronuclei the gDNA translocates into the intraluminal vesicles (future exosomes) of multivesicular bodies. (D) Both the gDNA content of the exosomes and their release are increased in tumor cells, especially upon the effect of therapeutic stress. (E) The gDNA may be transferred either in the lumen of sEVs and/or on the exofacial EV surface, or independently as a non-vesicular component. The gDNA content of sEVs might depend on their origin, like exocytosis of MVBs (D), amphisomes (F), or sEV discharge from en bloc released MVB-like EV clusters or migrasomes (G). The red frame indicates that all listed processes (i.e., migrasome formation, exosome secretion, amphisome exocytosis) may occur in both normal and tumor cells. (H) Uptake of the released sEVs by a recipient cell (right) may include receptor-mediated processes (characteristic of both normal and tumor cells, indicated by a red frame). (I) In the cytoplasm, the sEVs (or their components) may activate DNA recognition pathways e.g., cGAS/STING. (J) The EV-containing late endosomes may reach the nucleus. (K) Small vesicles (~200 nm) are also detectable in association with the nuclear membrane invaginations (red arrowheads in electron microscopy images of HT29 colorectal cancer cells). The origin of these vesicular structures has not been examined (scale bars: 500 nm). (L) The integration of gDNA into the recipient genome may require malfunction of the host DNA repair or onco-suppressor mechanisms (e.g., p53, and BRCA1). Many of the processes presented here have only been described in relation to cancers. Further studies are needed to demonstrate whether these occur in healthy cells.
BOX 2 Questions about the ability of sEVs to transport DNA.

It is generally accepted that sEVs carry higher amounts of double-stranded DNA as compared to the single-stranded one determined by using DNases that differentially recognize and digest the two types of DNA (such as Shrimp dsDNase and S1 nuclease) (16). Besides gDNA, the full mitochondrial genome is also identified in sEVs (34, 35). Furthermore, enhanced amounts of sEV-associated miDNA and tumor-specific gDNA have been described in the blood of cancer patients (35, 36). This latter property allows sEV-based identification of informative mutations from liquid biopsy applications, supporting the early detection and diagnosis of cancer as well as monitoring the treatment response (15). This established view is questioned by the suggestion that DNA released by cells is localized in DNasel-sensitive, non-vesicular structures (nucleosomes) released by the exocytosis of amphisomes (17). Although the release of other non-membranous, small (~30 nm) nanoparticles (i.e., exomers) has also been described, the authors have detected DNA in sEVs with cell-type-specific relative abundance (38). Furthermore, many earlier studies used DNase treatment for the examination of luminal (protected) DNA [e.g., (22, 31, 35, 39)]. Importantly Thakur et al. described that the large (>2.5 kb) double-stranded DNA fragments bind to the exofacial surface of exosomes, while the size of the luminal DNA fragments ranges from 100 bp to 2.5 kbp (16).

Although DNase treatment has confirmed the presence of luminal DNA content in sEVs in these studies, the question arises whether the DNA removed from the exofacial surface can indeed be classified as an artifact or the surface-associated DNA cargo is a native property of sEVs. This latter possibility was confirmed by an artificial selection pressure induced release of both DNA and DNA-binding proteins on the exofacial surface of sEVs in vitro (19). Based on this observation, it should be considered, that although DNase digestion removes potential foreign contaminats of sEVs, it may also eliminate the external, physiological, or pathophysiological DNA cargo from their exofacial vesicular surface.

Conceivably, we propose, that DNA from the microenvironment may also be transferred onto the sEV surface in the extracellular space (as part of a biomolecular corona). Thus, the genetic information carried by an sEV is not necessarily limited to a single EV donor cell.

The DNA cargo has been reported to be present only in a small proportion of sEVs (~10% in vitro, ~1% in vivo) (16, 24) suggesting that these vesicles are heterogeneous in this respect. This heterogeneity has also been confirmed using high-resolution iodixanol density gradients, discriminating high-density sEV fraction with enriched DNA content and a low-density sEV fraction which carried minor quantities of DNA (40). Because the sEV fraction separated by ultracentrifugation can be derived from multiple cell sources, we need to emphasize the heterogeneity both within sEVs of the same biogenic origin (e.g., exosome subpopulations) and sEVs released from different subcellular structures (i.e., MVBs, migrasomes, MVB-like EV clusters, Figure 1G, Box 1).

Probably, the packaging of the genomic nuclear content to sEVs cannot be simplified to a yes/no question. As detailed above, the gDNA content of the cytoplasmic region may be highly dependent on the existence of certain pathophysiological processes in the cell (e.g., nuclear membrane rupture and micronuclei formation) (23-25). It may lead to an increased gDNA content in MVB-originated sEVs (i.e., exosomes) (24, 27). The rapid change in gDNA content of exosomes by existing stress factors (e.g., effects of artificial selection or the pro-inflammatory microenvironment) have been observed (24, 29, 30), however, it is not known how similar stress conditions may regulate the non-MVB-originated sEV release. Presumably, these sEV release pathways may change dynamically upon exposure to various microenvironmental or therapy-induced stress factors.

Uptake and functions of sEV-gDNA in recipient cells

The tumor can be considered as an ecosystem in which both the cells and the subclonal populations cooperate with each other for acquiring space and resources from the host organism e.g., by sharing molecules with beneficial local and/or systemic effects (41-43). After the sEV-protected gDNA survives the release and is present in the blood or in other body fluids, the recipient cells may internalize it from the extracellular space [the known sEV uptake mechanisms are shown in Figure 1H, and were reviewed by McKelvey et al. (44)]. In fact, this process is not trivial, so the selective sEV uptake may be the first barrier to the spread of vesicle-carried genes with oncogenic potential. This selectivity shows the dependency on the type, oncogene status, and receptor repertoire of the recipient cells. Substantial differences in sEV uptake have been observed between normal epithelial cells compared to the tumorigenic variant of them (45), resulting in functional changes in the latter. This study also described robust sEV uptake by fibroblasts (compared to normal epithelial cells), although these fibroblasts lacked proper tumor suppressor mechanisms (45). In the absence of control fibroblasts, it is questionable whether increased sEV uptake was caused by cell-type dependency or other changes, associated with the malfunction of the tumor suppressor mechanisms (see below). The image is further refined by the observation that sEV adhesion to fibroblasts shows integrin receptor dependency resulting in a non-random (i.e., organotropit) metastasis formation (46). On the other hand, the sEV tetraspansin web also plays a primary role in selective target cell binding (i.e., formation of tetraspansin-integrin complexes) (47), which drives attention to the significance of proper donor and recipient cell selection when designing experiments. We have very limited knowledge about the uptake of unconventionally secreted sEVs [such as migrasomes and en bloc released MVB-like EV clusters (12, 13)]. Although uptake of migrasomes by recipient cells has been described in vitro (12), the details of this process are unknown.

The sEV-DNA uptake may be a robust process as it has been detected in ~16% of sEV-treated fibroblasts (48). The DNA, which enters the cytoplasm does not necessarily reach the nucleus but can activate cytoplasmic DNA recognition
receptors (Figure 1I). For instance, radiation or chemotherapy-induced, tumor-derived sEV-gDNA triggers antitumor immune response in dendritic cells by activating the cGAS/STING pathway (49, 50). In other cases, sEV-containing late endosomes have been shown to migrate to the invaginations of the nuclear envelope (51), where hypothetically, they can exchange genomic content between the donor sEVs and the recipient cell (Figure 1J). However, evidence for such an exchange is still lacking. The EV components may be released from the envelope invagination-associated late endosomes to the narrow space between the endosomal and nuclear membranes and might translocate into the nucleoplasm via nuclear pores (51). Ultrastructural studies of our research group detected single, small (<200 nm) vesicles within the nuclear invaginations of cancer cells (Figure 1K). However, the origin of these vesicles remains unknown, and obviously, their potential role in HGT should be investigated.

The last step of the oncogenic HGT is the process in which the transferred gDNA integrates into the recipient genome. Some factors have been described in prokaryotes and eukaryotes, which influence the rates of acceptance of the horizontally transferred genes, such as physical/biological properties of the acquired DNA (e.g., length, GC content, codon usage, epigenetic marks, and the complexity of interactions with other genes) as well as the location of genetic integration in the recipient genome (52, 53). However, the last phase of HGT between mammalian cells, especially in sEV-mediated processes has been less examined. Interestingly, genome engineering provides an opportunity for a more in-depth study of this process. The integration of donor DNA sequences at off-target double-strand breaks (DSBs) has been described during CRISPR-Cas9-assisted genome editing, which is known to be caused by an error-prone repair of a non-homologous end joining (NHEJ) pathway (54). The presence of bovine gDNA sequences were detectable in the genome of NIH-3T3 fibroblasts using a medium containing 10% fetal bovine serum. This effect was significantly reduced with the use of exosome-depleted but cfDNA-containing medium, suggesting a primary role of sEVs.

Consequently, during the study of the long-term effects of sEV-mediated HGT, the tumor evolutionary aspects should also be considered (see below and Box 3).

**The potential impact of sEV-mediated HGT in tumor evolution**

The tumor-associated sEV signaling, including the delivery of aberrantly released molecule packets (e.g., proteins, lipids, metabolites, coding- and non-coding nucleic acids) influences the evolutionary events of tumors by various, often parallel cellular processes (45, 80). Being the most cancer-specific component of this complex system, here we particularly focused on sEV-delivered gDNA in tumor evolution. Although oncogenic HGT is less known among tumor evolutionary biologists, the *in vitro* and *in vivo* results discussed here clearly indicate that this process is more than a theoretical phenomenon. The presence of clonal heterogeneity in cancer (81) suggests that the HGT-based cooperation among the admixed- or the spatially non-uniformly distributed subclones may be a rare event. Regarding its frequency, it must be emphasized that some phenomena associated with oncogenic HGT can be highly context-dependent (23, 24, 29, 30). Thus, the successful incorporation rate of sEV-mediated HGT may differ greatly depending on the imposed selection pressure. Cellular experiments modeling sEV-gDNA transfer under diverse selective pressures would be clinically relevant for mapping the transfer of resistance mutations between cells of
Conquering Muller’s ratchet and maintaining metabolic potential is particularly important for the survival of transmissible cancer cell lines that are able to spread across hosts and hence are being passed through the natural selection. One such transmissible cancer cell line is the Canine Venerable Tumor (CTVT), a sexually transmitted malignant cell line that affects dogs (73). CTVT is the oldest known living cancer with an estimated age of between 4,000 and 11,000 years (74-77). Since its emergence in Asia (77), CTVT has spread across the globe, infected millions of dogs, and most likely experienced the accumulation and homoplasmy of deleterious mtDNA mutations. To avoid genomic melt-downs and metabolic catastrophes, CTVT has been found to capture and incorporate host mtDNA multiple times, as well as to occasionally employ mtDNA recombination and re-assortment during its evolutionary history (78). Replacement of part of the cancer mtDNA genome with sequence from the host mtDNA has also been observed in another transmissible cancer cell line, the bivalve transmissible neoplasia (BTN) from Chile (79). Whether exosomes have been facilitating HGT in these unique cancer cell lines, remain to be answered and an intriguing research area to follow up.

By sEV-mediated HGT the recipient cells acquire adaptive benefits which can be manifested in increased proliferation, metastatic capacity, and forming tumor-like cells. However, these pioneering papers did not investigate HGT under intense therapeutic stress. The importance of therapy in an evolutionary context is highlighted by an observation about apoptotic EV-mediated HGT (6). [Here we note that although the apoptotic EVs arise as typical products of chemo- and radiotherapy, the transfer of full-length (3308 bp) sequences of H-ras (one of the examined genes in ref (6)) by sEVs was also described among living cells (31)]. When the incorporated DNA contains an advantageous mutation in the context of the treatment in question, it may become fixed, and it may spread among the recipient offspring cells. Thus, it may contribute to tumor evolution through several generations (6).

Accordingly, EV-mediated HGT may greatly affect the sensitivity profile of the cells in residual disease [often undetectable, small population of malignant cells which persist after therapy (85)]. Consequently, its inheritance to the recipient genome may determine the properties of the recurring tumor.

Furthermore, sEVs from the primary tumor may influence critical events of metastasis, such as the preparation of pre-metastatic microenvironment (46) and may induce the formation of potentially metastatic tumor cells at least partly via HGT (48). The metastatic spread by genetic material has been known for a long time (86), and it is consistent with Darwin’s pangenetic explanation (87). However, the role of sEVs in this process has only recently been studied and described. During the metastatic cascade, the phenotype of cancer cells shows dynamic changes, including epithelial-to-mesenchymal-, and mesenchymal-to-epithelial transitions (88, 89). The transition of BRCA1-KO fibroblasts to carcinoma-like cells (i.e., mesenchymal-to-epithelial transition) could be induced by sEV-gDNA (along with sEV-associated regulators) without preceding epithelial-to-mesenchymal transition (48). This suggests that the metastatic colonization is not exclusively due to the migration of primary tumor cells to metastatic sites [see the “seed to soil model” by Stephen Paget (90)], but it may also involve sEV-induced reprogramming of fibroblasts (48). In connection with this completely new phenomenon, further studies are needed to clarify as to whether carcinoma-like cells behave as tumor cells or as supporting microenvironment cells.

It is important to note that the above-mentioned studies examined the transition of one, or a few selected genes and their short functional effect with potential evolutionary benefits. However, parallel with beneficial genes, neutral or deleterious mutations are also conveyed into the recipient cells by HGT (5). Hypothetically, therefore, the role of oncogenic HGT in cancer...
may be twofold: transferring deleterious genes may accelerate the irreversible accumulation of mutations which ultimately cause a mutational meltdown. Secondly, it may also increase the genetic diversity required for rapid adaptation by transferring beneficial genes (see Box 3 for further details).

Conclusion

Presumably, under physiological conditions, mammalian sEV-mediated HGT may not be extensive, and it has a restricted evolutionary impact. However, we want to emphasize that cancer-associated alterations in DNA repair, sEV secretion and uptake, and functional integration of the transmitted genes might modify the typical range and effect of HGT. Considering that cancer is a special evolutionary system, with its fast-growing, closely spaced large populations of cells that have similar genomes, the effect of rare events is likely to be increased as compared to the physiologic non-tumorous conditions. Mapping of the complex HGT phenomenon and integrating the knowledge reviewed here into our thinking of cancer development and progression may help to better interpret genomic data and allow the development of more precise tumor evolution models.

Author contributions

GV, BÚ, EIB, TK, SS and ÁK wrote the manuscript. PI, ZT, IT and BM provided the critical revisions. All authors approved the final version of the manuscript for submission and approved it for publication.

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