Extracellular Vesicles (EVs)

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Extracellular Vesicles (EVs) represent a heterogeneous population of membranous cell-derived structures, including cargo-oriented exosomes and microvesicles. EVs are functionally associated with intercellular communication and play an essential role in multiple physiopathological conditions. Shedding of EVs is frequently increased in malignancies and their content, including proteins and nucleic acids, altered during carcinogenesis and cancer progression. EVs-mediated intercellular communication between tumor cells and between tumor and stromal cells can modulate, through cargo miRNA, the survival, progression, and drug resistance in cancer conditions. These consolidated suggestions and EVs' stability in bodily fluids have led to extensive investigations on the potential employment of circulating EVs-derived miRNAs as tumor biomarkers and potential therapeutic vehicles.

Keywords: microvesicle ; exosome ; miRNA ; nucleic acid cargo ; biomarkers ; diagnosis ; therapy ; clinical study ; cancer

1. General Characteristics and Nomenclature

The secretion of extracellular membranous particles is a widespread characteristic of both eukaryotic and prokaryotic cells. Extracellular Vesicles (EVs) are small membrane-bound vesicles that are secreted from numerous animal cell types, including a variety of tumor cells [1][2][3][4][5][6]. Several EVs subtypes have been proposed with names such as ectosomes, microvesicles, microparticles, exosomes, oncosomes, apoptotic bodies, and more. To date, the International Society for Extracellular Vesicles established that subtypes of EVs may be defined by physical characteristics such as size ("small EVs", sEVs, diameter < 100/150 nm and "medium/large EVs", m/lEVs, diameter > 200 nm), density (low, middle, high, with each range defined), biochemical composition (CD63+/CD81 EV-associated, Annexin A5-EVs), and description of conditions or cell of origin (podocyte EVs, hypoxic EVs, large oncosomes, apoptotic bodies) [7].

In general, the common nomenclature used in literature for EVs comprises exosomes (small EVs) and microvesicles (large EVs). Exosomes are released on multi-vesicular bodies (MVBs) fusion with the plasma membrane, they are ~30–150 nm in diameter and they have "cup-shaped" morphology when observed under transmission electron microscopy. Exosome biogenesis begins during the early-endosome maturation process. Early-endosomal membranes invaginate to create intraluminal vesicles (ILVs), thereby forming MVBs. The majority of MVBs are degraded by fusion with lysosomes; however, some populations of MVBs migrate towards and fuse with the plasma membrane, releasing their ILVs (now called exosomes) into the extracellular space [7][8][9]. Microvesicle biogenesis occurs via the direct outward blebbing and pinching of the plasma membrane releasing the nascent microvesicle into the extracellular space. Microvesicles are large EVs and very heterogeneous in size (~400–1000 nm) [7][10]. Consolidated data have largely demonstrated that EVs represent an important paracrine and endocrine mechanism for cell-to-cell communication, involved in the transfer of several different macromolecules including microRNAs (miRNAs), short non-coding RNAs with key roles in cellular regulation [11][12][13][14][15].

2. Functions in Tumoral Cells

Several mechanisms have been proposed to describe possible EVs/target cell interactions:

- EVs could interact directly with target cells by employing conventional ligand–receptor interactions.
- The membrane proteins of the EVs can be cut by specific proteases giving rise to fragments that could act as soluble ligands for membrane receptors on the target cells.
- EVs can merge with the target cell membrane by transferring cargo proteins and RNAs [16].

In particular, miRNAs transported within EVs can regulate the expression of protein-coding genes in a paracrine way by binding mRNAs in the target cells [17]. Accumulating evidence suggests that EVs play an important role in communication between tumors and the microenvironment; indeed, by transferring EVs cargo, tumor cells can alter the function of both local and distant normal cells, thereby promoting tumor progression, immune evasion, angiogenesis, and metastasis [14][18][19][21].

Due to the stability of EVs miRNAs in bodily fluids, and their functional association with cancer, circulating EVs miRNAs are now extensively investigated for their potential use as cancer biomarkers in diagnostics and several EVs isolation methods have been developed for the purification of exosomes and microvesicles from bodily fluids and/or cell culture media. Indeed, to date, several miRNAs contained within EVs have been consistently associated with cancer progression, metastasis, and aggressive tumor phenotypes [22][23][24][25][26][27].

The role of signal transducer renders EVs cargo a diagnostic tool and a fertile field of study in innovative therapy. Indeed, great interest has arisen in the possible clinical use of EVs in regenerative medicine thanks to their ability to act as paracrine mediators in cell–cell communication [28][29][30].

The release of EVs by cancer cells could play an important role also in protecting tumor cells from apoptosis and making them more resistant to chemotherapy by extruding apoptosis-inducing proteins and chemotherapy agents [31]. In such a background, EVs gained great attention as carriers for miRNA delivery to regulate target gene expression and improve cancer therapeutic strategies [32][33].

3. Profiling EVs-miRNAs

Numerous studies have been carried out to analyze miRNAs' involvement in human diseases through the development of a microarray platform, which made possible the global profiling of miRNAs. Significantly, in many cases, the classification of tumor phenotypes was more successful when the expression profile of miRNAs was used, rather than that of mRNAs. Although analysis of the miRNA profile in human tissues has been shown to have great potential as a disease marker, a less invasive method for tissue biopsy is certainly needed to make this analysis more affordable. In this regard, the identification of miRNA in body fluids attracted much interest. It prompted researchers in demonstrating that the miRNA profiles in the body fluids from patients were significantly different from the profiles in healthy subjects [34][35][36].

Moreover, these studies demonstrated that miRNAs in body fluids remained stable under various extreme conditions, such as boiling, a very low or high pH, repeated freeze–thaw, and storage at room temperature for a long time. Besides, thanks to the high sensitivity and speed of the new profiling techniques of molecular biology, today it is much easier to study the profile of the global miRNA level, rather than that of proteins or secreted metabolites [37][38].

Careful consideration of the EVs isolation method must be taken into account when interpreting study results. The most common methods are a series of differential centrifugation steps, ultrafiltration, chemical affinity purification, and Exoquick [39][40][41] (Table 1). As demonstrated in conditional medium from cell culture the purification methods of EVs may heavily influence the yield, purity, and integrity of RNA extracted from EVs [42][43]. Moreover, there are no reliable methods for purifying and discriminating between exosomes or microvesicles, and this could represent an important limitation in many studies. Indeed, even though both exosomes and microvesicles have been thought to share intercellular communicative ability and they present largely similar RNA profiles, a recent study points out that exosomes and microvesicles could have different biological roles. The study, which was performed using transiently transfected cells, showed that reported proteins and mRNA were successfully sealed in both types of vesicles but only microvesicles managed to convey reporter function to target cells [44][45][46].

Table 1. Pros and cons of the main exosome isolation and miRNA detection methods.

Experimental Procedures	Methodology	Advantages	Disadvantages
Exosome isolation	Ultracentrifugation	Bulk exosome purification is easy	Time-consuming, contaminating proteins
	Density gradient centrifugation	High purity exosome	Loss of exosomes, relies on user skill
	Ultrafiltration	Good exosome yield and quick isolation	Less purity
	Immunoaffinity methods	Use of exosome standard markers	Biological properties could be altered due to alterations in markers
	Polymer-based precipitation	Simple and easy procedure	Contamination and retention of polymer
miRNA detection	qRT-PCR	Multiplexed, quantitative, high sensitivity and specificity	Sensitive to contaminants, moderately labor-intensive, limited profiling
	Microarray	Comprehensive profiling, Multiplexed	Low specificity and sensitivity, expensive, relative quantitation
	Nanostring	Multiplexed, quantitative, high sensitivity and specificity	The emerging procedure, expensive, limited profiling
	Next-generation sequencing	Comprehensive profiling, Multiplexed, quantitative, high sensitivity, and specificity	Highly labor-intensive, expensive, requires bioinformatic analysis

To date, an elevated number of miRNAs have been associated with tissue expression patterns and linked to specific pathologies. Thus, their analysis can provide a large amount of information in a relatively short time. Tumor-related alterations in microRNA expression and function can reflect molecular processes of tumor onset and progression qualifying microRNAs as potential diagnostic and prognostic biomarkers. Current investigations show that miRNAs are detectable in different tissue types and a wide range of biological fluids, either free or trapped in circulating EVs [47][48].

Their abundance in circulating body fluids in conjunction with the feasibility in the recovery and characterization from liquid specimens render EVs cargo molecules promising diagnostic and prognostic biomarkers for cancers. The traditional enrichment and extraction processes of miRNA trapped inside EVs, are problematic due to the low concentration of miRNA within EVs and the lack of a standardized isolation method of EVs. Indeed, some authors, considering these difficulties, are skeptical about the possibility to translate EVs-miRNA analysis in the clinic. This is also justified by several unsolved problems, such as significant differences in the procedures for processing samples, methods of analysis, and high heterogeneity in results accumulated so far [49]. To solve the aforementioned problems, current studies are mainly focusing the attention on the needed improvements in methodology able to increase the reliability of EVs-miRNAs as a diagnostic tool [49]. A detailed critical discussion on methodology challenges is outside the purpose of this review and further details could be found in several studies cited in the text while advice about these aspects is present in MISEV2014 guidelines [7].

In biological samples, such as liquid biopsies, EVs-miRNAs can be analyzed through different techniques. Evaluation of plasma EVs-RNA profiles by RNA-seq individuated a variety of RNA species for their abundance, stability, and age/gender correlation as well as disease association. However, the experimental procedures can cause constitutive variations in EVs-miRNA profiles if using different methods for RNA extraction, sequencing library preparation, or gel size selection [50].

Several traditional methods have been employed for miRNA detection, including Northern blotting [51][52][53], microarray analysis [54] and quantitative polymerase chain reaction (qPCR) [55][56].

So far, qPCR is regarded as the gold standard. However, it has been associated with many limitations such as its time-consuming nature, its false-positive propensity, and difficulties in primer design. qPCR presents the main advantage of being highly sensitive and specific. The newly introduced next-generation sequencing (NGS) has recently proven to be extremely able to detect unknown miRNAs that the traditional methods, including the qPCR, are incapable of doing. miRNAs detection by NGS has progressed rapidly and is a promising field for applications in drug development [57][58] (Table 1).

Recent studies that have investigated miRNA profiling by NGS, with particular attention to the potential applicability on biofluids, aimed at comparing results with those obtained by qRT-PCR [59].

The high sensitivity of new molecular techniques suggests the adoption of accurate pre-analytical processing of EVs. Indeed, it has been suggested that EVs may associate with protein complexes and lipoproteins bound to free circulating miRNAs, suggesting the use of proteinase K and RNase A before the analysis of miRNA-EVs to remove the extra-vesicular contamination source. Indeed, the proteinase and RNase treatment can reduce the relative quantity value by up to 70% for different miRNAs [60].

The utilization of new technologies producing a huge amount of data generates new problems concerning statistical analysis and data interpretation. It could be a difficult procedure to select the miRNA profile that effectively plays a role in tumor development and progression, without incurring bias. A single miRNA can target many mRNAs and modulate many proteins involved in different cancer-related pathways. Moreover, some miRNAs can act as both oncogenes and oncosuppressors, according to the biological context, and this makes it more complicated to unveil their effective role. To identify potential biological functions and potential targets of miRNAs several software programs such as TargetScan, miRbase, miRWalk, DIANA-microT, and TarBase have been described, and for more information about this software, we suggest the reading of specific publications [28][29][61][62].

References

1. Budnik, V.; Ruiz-Canada, C.; Wendler, F. Extracellular vesicles round off communication in the nervous system. Nat. Rev. Neurosci. 2016, 17, 160–172.
2. Lai, R.C.; Yeo, R.W.; Lim, S.K. Mesenchymal stem cell exosomes. Semin. Cell Dev. Biol. 2015, 40, 82–88.
3. Zhao, C.; Gao, F.; Weng, S.; Liu, Q. Pancreatic cancer and associated exosomes. Cancer Biomark. 2017, 20, 357–367.
4. Yu, D.D.; Wu, Y.; Shen, H.Y.; Lv, M.M.; Chen, W.X.; Zhang, X.H.; Zhong, S.L.; Tang, J.H.; Zhao, J.H. Exosomes in development, metastasis and drug resistance of breast cancer. Cancer Sci. 2015, 106, 959–964.
5. Srivastava, A.; Amreddy, N.; Razaq, M.; Towner, R.; Zhao, Y.D.; Ahmed, R.A.; Munshi, A.; Ramesh, R. Exosomes as Theranostics for Lung Cancer. Adv. Cancer Res. 2018, 139, 1–33. [Google Scholar]
6. Lin, L.Y.; Yang, L.; Zeng, Q.; Wang, L.; Chen, M.L.; Zhao, Z.H.; Ye, G.D.; Luo, Q.C.; Lv, P.Y.; Guo, Q.W.; et al. Tumor-originated exosomal lncUEGC1 as a circulating biomarker for early-stage gastric cancer. Mol. Cancer 2018, 17, 84.
7. Thery, C.; Witwer, K.W.; Aikawa, E.; Alcaraz, M.J.; Anderson, J.D.; Andriantsitohaina, R.; Antoniou, A.; Arab, T.; Archer, F.; Atkin-Smith, G.K.; et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles 2018, 7, 1535750.
8. Hessvik, N.P.; Llorente, A. Current knowledge on exosome biogenesis and release. Cell. Mol. Life Sci. 2018, 75, 193–208.
9. Van Niel, G.; D'Angelo, G.; Raposo, G. Shedding light on the cell biology of extracellular vesicles. Nat. Rev. Mol. Cell Biol. 2018, 19, 213–228.
10. Tricarico, C.; Clancy, J.; D'Souza-Schorey, C. Biology and biogenesis of shed microvesicles. Small GTPases 2017, 8, 220–232.
11. Desrochers, L.M.; Bordeleau, F.; Reinhart-King, C.A.; Cerione, R.A.; Antonyak, M.A. Microvesicles provide a mechanism for intercellular communication by embryonic stem cells during embryo implantation. Nat. Commun. 2016, 7, 11958.
12. Lazaro-Ibanez, E.; Lasser, C.; Shelke, G.V.; Crescitelli, R.; Jang, S.C.; Cvjetkovic, A.; Garcia-Rodriguez, A.; Lotvall, J. DNA analysis of low- and high-density fractions defines heterogeneous subpopulations of small extracellular vesicles based on their DNA cargo and topology. J. Extracell. Vesicles 2019, 8, 1656993.
13. Jeppesen, D.K.; Fenix, A.M.; Franklin, J.L.; Higginbotham, J.N.; Zhang, Q.; Zimmerman, L.J.; Liebler, D.C.; Ping, J.; Liu, Q.; Evans, R.; et al. Reassessment of Exosome Composition. Cell 2019, 177, 428–445.e18.
14. Desrochers, L.M.; Antonyak, M.A.; Cerione, R.A. Extracellular Vesicles: Satellites of Information Transfer in Cancer and Stem Cell Biology. Dev. Cell 2016, 37, 301–309.
15. de Rie, D.; Abugessaisa, I.; Alam, T.; Arner, E.; Arner, P.; Ashoor, H.; Astrom, G.; Babina, M.; Bertin, N.; Burroughs, A.M.; et al. An integrated expression atlas of miRNAs and their promoters in human and mouse. Nat. Biotechnol. 2017, 35, 872–878.
16. Mathivanan, S.; Ji, H.; Simpson, R.J. Exosomes: Extracellular organelles important in intercellular communication. J. Proteom. 2010, 73, 1907–1920.

17. Terlecki-Zaniewicz, L.; Lammermann, I.; Latreille, J.; Bobbili, M.R.; Pils, V.; Schosserer, M.; Weinmullner, R.; Dellago, H.; Skalicky, S.; Pum, D.; et al. Small extracellular vesicles and their miRNA cargo are anti-apoptotic members of the se nescence-associated secretory phenotype. Aging 2018, 10, 1103–1132.
18. Angelucci, A.; D'Ascenzo, S.; Festuccia, C.; Gravina, G.L.; Bologna, M.; Dolo, V.; Pavan, A. Vesicle-associated urokina se plasminogen activator promotes invasion in prostate cancer cell lines. Clin. Exp. Metastasis 2000, 18, 163–170.
19. Bebelman, M.P.; Smit, M.J.; Pegtel, D.M.; Baglio, S.R. Biogenesis and function of extracellular vesicles in cancer. Phar macol. Ther. 2018, 188, 1–11.
20. Kalluri, R. The biology and function of exosomes in cancer. J. Clin. Investig. 2016, 126, 1208–1215.
21. Mashouri, L.; Yousefi, H.; Aref, A.R.; Ahadi, A.M.; Molaei, F.; Alahari, S.K. Exosomes: Composition, biogenesis, and me chanisms in cancer metastasis and drug resistance. Mol. Cancer 2019, 18, 75.
22. Kamerkar, S.; LeBleu, V.S.; Sugimoto, H.; Yang, S.; Ruivo, C.F.; Melo, S.A.; Lee, J.J.; Kalluri, R. Exosomes facilitate th erapeutic targeting of oncogenic KRAS in pancreatic cancer. Nature 2017, 546, 498–503.
23. Agrahari, V.; Agrahari, V.; Burnouf, P.A.; Chew, C.H.; Burnouf, T. Extracellular Microvesicles as New Industrial Therape utic Frontiers. Trends Biotechnol. 2019, 37, 707–729.
24. Susa, F.; Limongi, T.; Dumontel, B.; Vighetto, V.; Cauda, V. Engineered Extracellular Vesicles as a Reliable Tool in Can cer Nanomedicine. Cancers 2019, 11, 1979.
25. Lane, R.E.; Korbie, D.; Trau, M.; Hill, M.M. Purification Protocols for Extracellular Vesicles. Methods Mol. Biol. 2017, 16 60, 111–130. [Google Scholar]
26. Kowal, J.; Arras, G.; Colombo, M.; Jouve, M.; Morath, J.P.; Primdal-Bengtson, B.; Dingli, F.; Loew, D.; Tkach, M.; Thery, C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subt ypes. Proc. Natl. Acad. Sci. USA 2016, 113, E968–E977.
27. Perez-Gonzalez, R.; Gauthier, S.A.; Kumar, A.; Saito, M.; Saito, M.; Levy, E. A Method for Isolation of Extracellular Vesi cles and Characterization of Exosomes from Brain Extracellular Space. Methods Mol. Biol. 2017, 1545, 139–151. [Goo gle Scholar]
28. Di Rocco, G.; Baldari, S.; Toietta, G. Towards Therapeutic Delivery of Extracellular Vesicles: Strategies for In Vivo Track ing and Biodistribution Analysis. Stem Cells Int. 2016, 2016, 5029619.
29. Fuster-Matanzo, A.; Gessler, F.; Leonardi, T.; Iraci, N.; Pluchino, S. Acellular approaches for regenerative medicine: On the verge of clinical trials with extracellular membrane vesicles? Stem Cell Res. Ther. 2015, 6, 227.
30. Riazifar, M.; Pone, E.J.; Lotvall, J.; Zhao, W. Stem Cell Extracellular Vesicles: Extended Messages of Regeneration. An nu Rev. Pharmacol. Toxicol. 2017, 57, 125–154.
31. Abid Hussein, M.N.; Boing, A.N.; Sturk, A.; Hau, C.M.; Nieuwland, R. Inhibition of microparticle release triggers endothe lial cell apoptosis and detachment. Thromb. Haemost. 2007, 98, 1096–1107.
32. Andaloussi, S.E.L.; Mager, I.; Breakefield, X.O.; Wood, M.J. Extracellular vesicles: Biology and emerging therapeutic o pportunities. Nat. Rev. Drug Discov. 2013, 12, 347–357.
33. Kosaka, N.; Takeshita, F.; Yoshioka, Y.; Hagiwara, K.; Katsuda, T.; Ono, M.; Ochiya, T. Exosomal tumor-suppressive mi croRNAs as novel cancer therapy: "Exocure" is another choice for cancer treatment. Adv. Drug Deliv. Rev. 2013, 65, 37 6–382.
34. Kwak, Y.H.; Kwak, D.K.; Kim, N.Y.; Kim, Y.J.; Lim, J.S.; Yoo, J.H. Significant changes in synovial fluid microRNAs after high tibial osteotomy in medial compartmental knee osteoarthritis: Identification of potential prognostic biomarkers. PLo S ONE 2020, 15, e0227596.
35. Gholami, D.; Salman Yazdi, R.; Jami, M.S.; Ghasemi, S.; Sadighi Gilani, M.A.; Sadeghinia, S.; Teimori, H. The expressi on of Cysteine-Rich Secretory Protein 2 (CRISP2) and miR-582-5p in seminal plasma fluid and spermatozoa of infertile men. Gene 2020, 730, 144261.
36. Lee, H.; Groot, M.; Pinilla-Vera, M.; Fredenburgh, L.E.; Jin, Y. Identification of miRNA-rich vesicles in bronchoalveolar l avage fluid: Insights into the function and heterogeneity of extracellular vesicles. J. Control. Release 2019, 294, 43–52.
37. Cho, S.; Yang, H.C.; Rhee, W.J. Simultaneous multiplexed detection of exosomal microRNAs and surface proteins for prostate cancer diagnosis. Biosens. Bioelectron. 2019, 146, 111749.
38. Wang, T.; Turko, I.V. Proteomic Toolbox to Standardize the Separation of Extracellular Vesicles and Lipoprotein Particle s. J. Proteome Res. 2018, 17, 3104–3113.
39. Petit, I.; Levy, A.; Aberdam, D. Purification of Extracellular Microvesicles Secreted by Dermal Fibroblasts. Methods Mol. Biol. 2020, 2154, 63–72. [Google Scholar]

40. Iliuk, A.; Wu, X.; Li, L.; Sun, J.; Hadisurya, M.; Boris, R.S.; Tao, W.A. Plasma-Derived Extracellular Vesicle Phosphoproteomics through Chemical Affinity Purification. J. Proteome Res. 2020, 19, 2563–2574.

41. Hu, J.L.; Wang, W.; Lan, X.L.; Zeng, Z.C.; Liang, Y.S.; Yan, Y.R.; Song, F.Y.; Wang, F.F.; Zhu, X.H.; Liao, W.J.; et al. CAFs secreted exosomes promote metastasis and chemotherapy resistance by enhancing cell stemness and epithelial-mesenchymal transition in colorectal cancer. Mol. Cancer 2019, 18, 91.

42. Pachler, K.; Lener, T.; Streif, D.; Dunai, Z.A.; Desgeorges, A.; Feichtner, M.; Oller, M.; Schallmoser, K.; Rohde, E.; Gimona, M. A Good Manufacturing Practice-grade standard protocol for exclusively human mesenchymal stromal cell-derived extracellular vesicles. Cytotherapy 2017, 19, 458–472.

43. Mateescu, B.; Kowal, E.J.; van Balkom, B.W.; Bartel, S.; Bhattacharyya, S.N.; Buzas, E.I.; Buck, A.H.; de Candia, P.; Chow, F.W.; Das, S.; et al. Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - an ISEV position paper. J. Extracell. Vesicles 2017, 6, 1286095.

44. Chen, M.; Xu, R.; Rai, A.; Suwakulsiri, W.; Izumikawa, K.; Ishikawa, H.; Greening, D.W.; Takahashi, N.; Simpson, R.J. Distinct shed microvesicle and exosome microRNA signatures reveal diagnostic markers for colorectal cancer. PLoS ONE 2019, 14, e0210003.

45. Kanada, M.; Bachmann, M.H.; Hardy, J.W.; Frimannson, D.O.; Bronsart, L.; Wang, A.; Sylvester, M.D.; Schmidt, T.L.; Kaspar, R.L.; Butte, M.J.; et al. Differential fates of biomolecules delivered to target cells via extracellular vesicles. Proc. Natl. Acad. Sci. USA 2015, 112, E1433–E1442.

46. Ji, W.; Sun, B.; Su, C. Targeting MicroRNAs in Cancer Gene Therapy. Genes 2017, 8, 21.

47. Fauth, M.; Hegewald, A.B.; Schmitz, L.; Krone, D.J.; Saul, M.J. Validation of extracellular miRNA quantification in blood samples using RT-qPCR. FASEB Bioadv. 2019, 1, 481–492.

48. Cisilotto, J.; do Amaral, A.E.; Rosolen, D.; Rode, M.P.; Silva, A.H.; Winter, E.; da Silva, T.E.; Fischer, J.; Matiollo, C.; Rateke, E.C.M.; et al. MicroRNA profiles in serum samples from Acute-On-Chronic Liver Failure patients and miR-25-3p as a potential biomarker for survival prediction. Sci. Rep. 2020, 10, 100.

49. Chen, B.Y.; Sung, C.W.; Chen, C.; Cheng, C.M.; Lin, D.P.; Huang, C.T.; Hsu, M.Y. Advances in exosomes technology. Clin. Chim. Acta 2019, 493, 14–19.

50. Yuan, T.; Huang, X.; Woodcock, M.; Du, M.; Dittmar, R.; Wang, Y.; Tsai, S.; Kohli, M.; Boardman, L.; Patel, T.; et al. Plasma extracellular RNA profiles in healthy and cancer patients. Sci. Rep. 2016, 6, 19413.

51. Akmal, M.; Baig, M.S.; Khan, J.A. Suppression of cotton leaf curl disease symptoms in Gossypium hirsutum through over expression of host-encoded miRNAs. J. Biotechnol. 2017, 263, 21–29.

52. Kim, S.W.; Li, Z.; Moore, P.S.; Monaghan, A.P.; Chang, Y.; Nichols, M.; John, B. A sensitive non-radioactive northern blot method to detect small RNAs. Nucleic Acids Res. 2010, 38, e98.

53. Varallyay, E.; Burgyan, J.; Havelda, Z. MicroRNA detection by northern blotting using locked nucleic acid probes. Nat. Protoc. 2008, 3, 190–196.

54. Li, W.; Ruan, K. MicroRNA detection by microarray. Anal. Bioanal. Chem. 2009, 394, 1117–1124.

55. Niu, J.; Zhang, L.; Qiu, H.; Wu, Y.; Wang, Z.; Zai, Y.; Liu, L.; Qu, J.; Kang, K.; Gou, D. An improved method for detecting circulating microRNAs with S-Poly(T) Plus real-time PCR. Sci. Rep. 2015, 5, 15100. [Google Scholar]

56. Mohammadi-Yeganeh, S.; Paryan, M.; Mirab Samiee, S.; Soleimani, M.; Arefian, E.; Azadmanesh, K.; Mostafavi, E.; Mahdian, R.; Karimipoor, M. Development of a robust, low cost stem-loop real-time quantification PCR technique for miRNA expression analysis. Mol. Biol. Rep. 2013, 40, 3665–3674.

57. Ohno, S.; Drummen, G.P.; Kuroda, M. Focus on Extracellular Vesicles: Development of Extracellular Vesicle-Based Therapeutic Systems. Int. J. Mol. Sci. 2016, 17, 172.

58. Wang, J.; Zheng, Y.; Zhao, M. Exosome-Based Cancer Therapy: Implication for Targeting Cancer Stem Cells. Front. Pharmacol. 2016, 7, 533.

59. Matullo, G.; Naccarati, A.; Pardini, B. MicroRNA expression profiling in bladder cancer: The challenge of next-generation sequencing in tissues and biofluids. Int. J. Cancer 2016, 138, 2334–2345.

60. Endzelins, E.; Berger, A.; Melne, V.; Bajo-Santos, C.; Sobolevska, K.; Abols, A.; Rodriguez, M.; Santare, D.; Rudnickiha, A.; Lietuvietis, V.; et al. Detection of circulating miRNAs: Comparative analysis of extracellular vesicle-incorporated miRNAs and cell-free miRNAs in whole plasma of prostate cancer patients. BMC Cancer 2017, 17, 730.

61. Bortolomeazzi, M.; Gaffo, E.; Bortoluzzi, S. A survey of software tools for microRNA discovery and characterization using RNA-seq. Brief. Bioinform. 2019, 20, 918–930.

62. Paraskevopoulou, M.D.; Vlachos, I.S.; Hatzigeorgiou, A.G. DIANA-TarBase and DIANA Suite Tools: Studying Experimentally Supported microRNA Targets. Curr. Protoc. Bioinform. 2016, 55, 12.14.1–12.14.18.