Chapter

CRISPR Cas/Exosome Based Diagnostics: Future of Early Cancer Detection

P.P. Mubthasima, Kaumudi Pande, Rajalakshmi Prakash and Anbarasu Kannan

Abstract

Trending and Thriving, CRISPR/Cas has expanded its wings towards diagnostics in recent years. The potential of evading off targeting has not only made CRISPR/Cas an effective therapeutic aid but also an impressive diagnostic tool for various pathological conditions. Exosomes, 30 - 150nm sized extracellular vesicle present and secreted by almost all type of cells in body per se used as an effective diagnostic tool in early cancer detection. Cancer being the leading cause of global morbidity and mortality can be effectively targeted if detected in the early stage, but most of the currently used diagnostic tool fails to do so as they can only detect the cancer in the later stage. This can be overcome by the use of combo of the two fore mentioned diagnostic aids, CRISPR/Cas alongside exosomes, which can bridge the gap compensating the cons. This chapter focus on two plausible use of CRISPR/Cas, one being the combinatorial aid of CRISPR/Cas and Exosome, the two substantial diagnostic tools for successfully combating cancer and other, the use of CRISPR in detecting and targeting cancer exosomes, since they are released in a significant quantity in early stage by the cancer cells.

Keywords: CRISPR/Cas, exosomes, cancer, Cas12a, Cas13a, exosomal noncoding RNAs

1. Introduction

1.1 Cancer

Amongst the non-communicable diseases, cancer remains to be the major cause of morbidity and mortality globally. As per the GLOBOCAN index 2020, 19.3 million new cancer cases have been reported with around 10.0 million cancer deaths in 2020. Amongst the top 10 cancers, Lung cancer is considered leading cause of mortality with 18.0% of the total cancer deaths, followed by colorectal cancer with the death rate 9.4%, liver with the mortality rate of 8.3%, stomach with 7.7%, and female breast cancer with the death rate of 6.9% (**Figure 1**) [1]. Cancer, by definition is considered as a condition characterized with uncontrolled division of particular cell type in a definite site anywhere in the body as an aftermath of various triggering factors technically termed as Carcinogens, which have the ability to convert proto - oncogenes to Oncogenes causing cancer. Cancer cells can

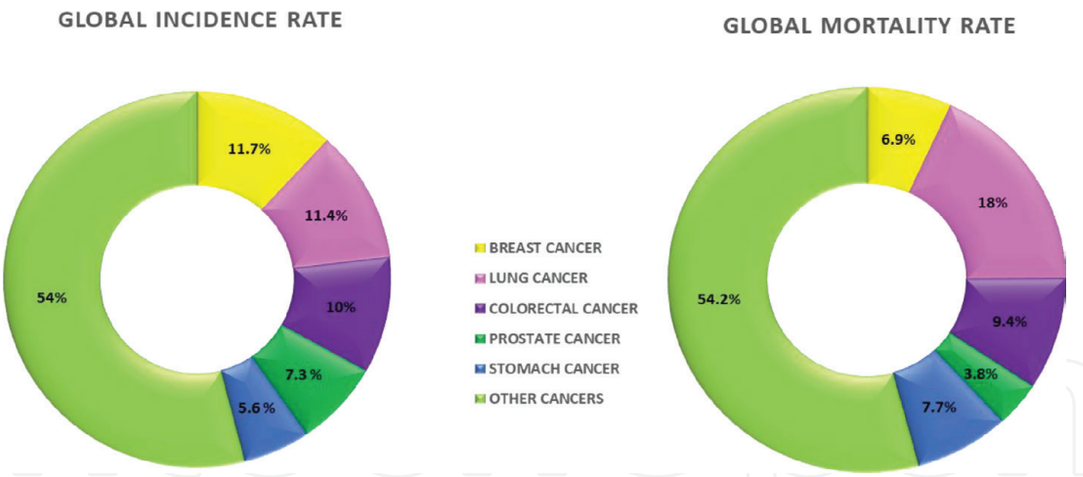

Figure 1.
Global incidence rate and death rate.

divert metabolites into anabolic pathways to support their rapid proliferation and to accumulate the cellular building blocks required for tumor growth and differ morphologically from other normal cells. It is reported that only around 5 – 10% total cancer is caused due to genetic defects such as *BRCA1* and *BRCA2* gene mutation in case of breast and ovarian cancer. Whereas, the other 90% is due to external factors such as exposure to potent carcinogens, lifestyle factors such as diet, stress, obesity, infections etc. [2–5].

WHO has broadly classified these carcinogens into physical (UV and other ionizing radiations), Chemical (alcohol, tobacco smoke, aflatoxins and various laboratory chemicals), Biological (Viruses such as Human Papilloma virus, hepatitis B virus, HIV etc., bacteria such as *Helicobacter pylori*, *Mycobacterium tuberculosis* etc., and parasites such as *Schistosoma haematobium*) and environmental (Air pollutants) factors [6]. The uncontrolled growth of cells when occur in a solid tissue such as in an organ or muscle are termed as tumors. All tumors not necessarily be cancerous, the tumors are further classified into benign tumors, which are noncancerous, devoid of spreading and malignant tumors, which are the cancerous ones that have the potential to spread to a distant organ via blood stream [7]. Cancer cells are known to invade adjoining parts of the body and spread to other organs to form the secondary tumor and this process is termed as metastasis, the primary cause of death from cancer.

Histologically, WHO has broadly classified cancers into following main categories viz.... Carcinoma (Cancer of epithelial tissue), Sarcoma (cancer of connective tissue), Myeloma (cancer of plasma cells of bone marrow), Lymphoma (cancer of lymphatic system), Leukemia (liquid cancer or cancer of blood), melanoma (cancer of pigment cells) and mixed types. Carcinoma accounts for around 180 – 90% of all cancer cases and are further sub divided into two categories such as Adenocarcinoma and squamous cell carcinoma [8, 9].

TNM (Tumor node metastasis) classification system, created in 1958 by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer control (UICC) is yet another widely used system that classifies malignant tumor based on tumor spreading. It usually scores the size of the primary tumor (T), degree of spreading to the lymph node (N) and the presence of distant metastasis (M). Based on different combinations of T, N and M, cancer has been categorized into different stages 0 – IV for the aid of clinicians to establish the anatomic extent of infection. The stage 0 represents carcinoma in situ with the combination Tis, N0, M0 and are considered noncancerous but with the possibility of becoming one. Whereas, Stage I represents localized cancer with the TNM combination

of T1-T2, N0, M0. Stage II is referred to locally advanced cancer with early stages and combination of T2-T4, N0, M0. While Stage III is characterized with locally advanced cancer, late stages with the combination T1-T4, N1-N3, M0, here the cancer would have been progressed with respect to the size of the tumor as well as it would have been spread to the adjacent lymph nodes. The stage IV is considered as the most severe stage, which is metastatic cancer with the combination, T1-T4, N1-N3, M1 [7, 10–12]. It is reported by that the above staging system is associated with severity of the disease and the survival rate of the patients, which is indirectly proportional i. e., higher the cancer stage so will be the severity of the disease and lesser will be the survival rate. For instance, 5-year survival rate of colorectal carcinoma at stage I is around 74% whereas, at stage IV it is only of 5% [10].

The signs and symptoms of cancer includes loss of appetite, extreme fatigue, pain in certain areas, persistent coughing, sudden loss of weight, blood in sputum, urine or stool, lumps on neck, breast, testicle etc. that does not hurt, changes in skin coloration, texture in certain areas etc. These fore said symptoms may not all ways point towards cancer, it could be due to any other pathological conditions too, but are the ones that should not be ignored [13, 14]. Currently there are several diagnostic tools in use for the detection of cancer such as laboratory testing of blood and urine for unusual blood count and for the detection of cancer biomarkers such as CA 125, CA 19-9, CA-15-3, CD117, CD19, CD 20, HE4, alpha-fetoproteins (AFP), bladder tumor antigen (BTA) etc. [15, 16]. Noninvasive Imagining tests includes CT scan, X-ray, mammography, ultrasound, Positron emission tomography (PET) etc. and more invasive method of biopsy which is considered as the golden standard for cancer diagnosis. However these diagnostics tools are not devoid of their own cons as most of them only detect cancer in its later stages leading to poor treatment efficiency [17].

The available treatment options for cancer includes surgical removal of solid tumor, Chemotherapy, Radiation therapy, Gene therapy, hormone therapy, immuno therapy, bone marrow transplantation, targeted drug therapy etc. Based on the severity, resistance of the cancer cells towards any of the above mentioned therapeutic options and in order to avoid cancer relapse, a combination of the fore said therapies will be used as an adjuvant therapy. For instance surgical removal in combination with chemotherapy and radiation therapy are practiced in order to avoid recurrence rate of the cancer [18].

1.2 Exosomes

Exosomes, the nanosized extracellular vesicles ranging 30 - 150 nm, are known to be secreted by all most all types of cells into the extracellular space and are present in all body fluids viz.... blood, tears, saliva, sputum, pleural fluid/effusions, cerebrospinal fluid (CSF), breast milk, amniotic fluid, semen, urine etc. [19, 20]. First discovered in the year 1983 by Stahl and Johnstone independently in reticulocytes, these extracellular vesicles were later termed as "Exosomes" by Rose Johnstone in the year 1987 [21–23]. The exosomes are reported to be encompass bimolecular components such as proteins, lipid, DNA, RNA (both coding as well as non-coding), metabolites and various enzymes etc. recapitulating the parent cell. Morphologically, exosomes are cup shaped extracellular vesicles where the central lumen composing the cargo of variety of fore mentioned biomolecules will be surrounded by the lipid bilayer structure. Accounting for the presence of these cargo, these nano scaled endocytic vesicles has engrossed plethora of attention amongst the scientific community around the globe, for its remarkable role as an efficient diagnostic tool [24–26].

The exosomes are known to be produced more in cancer cells than that of normal cells. The tumor derived exosomes are mainly involved in the cell–cell

communication between cancer cells with both adjacent as well as distant cells as they gets secreted to the extracellular space and travel to a longer distant organ and tissue via blood stream facilitating cancer proliferation, Metastasis, drug resistance and immunomodulation. Apart from being involved in the dynamic crosstalk between the cells exosomes also proves to be an ideal drug delivery system with benefits such as specificity, safety and stability, since they are small and native to animals, they are able to avoid recognition and premature degradation by body's immune defense mechanism. In recent years, exosomes are emerging as a promising biomarker tool as they carry specific genetic information and influence tumor growth and progression [27, 28].

1.3 CRISPR/Cas

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR associated endonuclease), a remarkable genome editing tool, has garnered plethora of interest of researchers in the field of modern gene therapy. Ever since the discovery of CRISPR locus in *E. coli* in the year 1987, several molecular biologists have shown tremendous interest and effort in characterizing and developing this wonder technique whereas, the noble prize for the development of this gene editing tool was bagged by Emmanuelle Charpentier and Jennifer Doudna in the year 2020 [29].

The CRISPR/Cas system is thought to be evolved from the prokaryotes such as bacteria and archaea as part of their adaptive immune system for combating viral infections. Precisely, they use a fragment of previously invaded viral genome called "spacer" as a source to memorize and defend any future attack by destroying the DNA from the similar viral particle, in association of Cas protein, which is an endonuclease enzyme, a molecular scissor to cut the double stranded DNA at a specific location on the target genome. It is reported that the CRISPR/Cas array is made of AT rich leader sequence, which is fenced by a set of Cas genes encoding the Cas proteins. The CRISPR/Cas is known to act through inducing site-specific DNA double stranded breaks and are known to surpass the other genome editing tools such as meganucleases, zinc finger nucleases (ZFNs), transcription activator like effectors (TALENs) etc., in being more precise, faster, efficient and inexpensive.

To brief out the conventional mechanism through which CRISPR/Cas executes their genome editing the following mechanism of gene editing pertaining to involvement of CRISPR/Cas9 has been described as follows, the CRISPR/Cas9 system is said to be RNA guided DNA targeting endonuclease system, which works through sequence specific manner. The mechanism of CRISPR/Cas9 based genome editing has been divided into three main stages viz.... I) DNA acquisition from the invading phage particle (adaptation). II) Biogenesis of CrRNA or CRISPR/Cas assembly formation and III) target DNA annihilation or interference of the target DNA and Insertion of desired gene sequence (i. e., either knock out or knock in). briefly, a small fragment or scrap of foreign invading genome termed spacer gets incorporated genomic CRISPR array and gets transcribed during the process of adaptation or in the 1st stage leading to the synthesis of crRNA, which in turn gets bound to Cas endonucleases enabling specificity towards the target.

The advantage of CRISPR/Cas system over the other gene editing platforms are, it has the property of multiplicity, simple, easy to prepare and use, as only 20 nucleotides in the guide RNA needs to be changed in order to retarget the Cas in CRISPR/Cas which in case of ZFNs and TALENs goes beyond 500-1500 base pairs. The CRISPR/Cas system can use multiple guide RNAs for targeting multiple target sites simultaneously in the same cell at the same time. This justifies the property of multiplicity of CRISPR/Cas system [29–35].

2. Types of CRISPR/Cas systems and their applications

Based on the type Cas protein involved and the complexity of components the CRISPR/Cas system is divided into two classes viz.... Class I and Class II and are further classified into six types which are in turn categorized into 22 sub types. The class 1 classification that comprises of type I, III and IV are characterized to be containing the involvement of multiple Cas proteins are whereas, class II classification are reported to comprise types II, V and VI where, only one effector Cas protein will be associated alongside the CRISPR array of processed guide RNA or in other words crRNA. Accounting for their nature of simplicity, the types involved under Class II Classification of CRISPR/Cas are reported to be the easiest in using for effective genome edited and manipulation of nucleic acids devoid of cells. Apart from being involved in gene editing the types of CRISPR/Cas systems derived from Class2 classification i. e., type II, V and VI are said to be instrumental in developing competent diagnostic platform of disease detection [36]. Brief representation of the fore said classification along with their application pertaining to class II CRISPR/Cas is as follows, **Figure 2**.

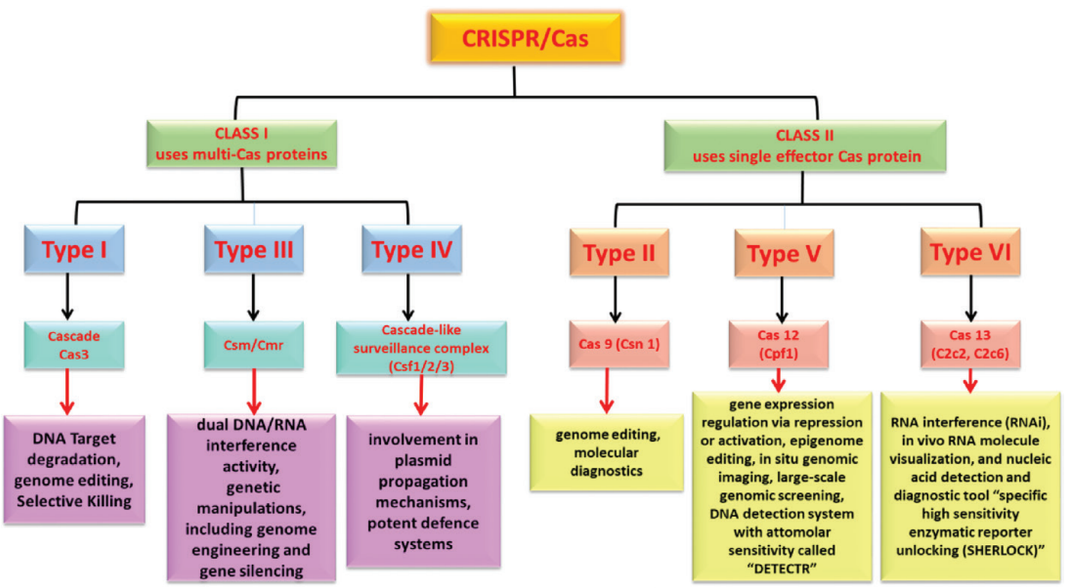

Figure 2.
Classification of CRISPR/Cas system with their emphasized application.

3. Exosomes in diagnosis

As mentioned prior in the introduction, exosomes are the nanosized form of extracellular vesicle of 30-150 nm size synthesized through a endosomal pathway via involvement of early, late endosomes and multi vesicular bodies (MVB) and are known to encompass several proteins lipids, nucleic acids etc. as their cargo. The exosomes are said to be involved in several biological processes such as, these are involved in cell–cell communication by the virtue of its cargo of fore mentioned compounds in the introduction both in physiological as well as pathological conditions. In pathological conditions including cancer, the cancer cell derived exosomes are reported to be synthesized or secreted in larger quantity than that from the normal cells, these cancer cell derived exosomes are considered key players in tumor growth and metastasis and are thought to be involved in stimulation of immune response. Apart from these, the exosomes are also suggested to be the part of dynamic cross talk between cancer cells and surrounding normal cells such as

fibroblasts, endothelial cells, mesenchymal cells etc., and are considered to play an important role mediating resistance towards therapy [26, 27].

The biomarkers are any biochemical component of the body, whose presence can be used as an indication of certain pathological condition. A cancer biomarker are known to represent any molecule or a process that shows the existence of tumor or cancer in the body. Cancer exosomes are one such components, which in recent years have gained tremendous importance as a liquid biopsy tool as these exosomes are exact representation of their parent cell in terms of their cargo and reflects the altered state of the parent cell. The cancer exosomes are known to contribute to cancer progression via enhancement of intercellular transfer of their cargo within the tumor microenvironment. These minimally invasive biomarkers are more convenient over the conventional tissue biopsy involving surgery in being highly sensitive and specificity and are involved in early detection of cancer as these exosomes are required for the metastatic niche formation that can be accomplished by their release to the circulatory system from where they can be detected [26, 37, 38].

Isolation and characterization of exosomes are consider a very crucial step in diagnostics and biomarker development. Several techniques have been employed for an efficient isolation of these exosomes which includes classical ultracentrifugation (differential ultracentrifugation), precipitation based isolation (exosome isolation kits), using size exclusion chromatography, filtration based isolation, using immunomagnetic isolation method etc. Followed by their isolation these exosomes can be characterized for their number, size and zeta potential value by using instruments such as nano trafficking analysis (NTA), Dynamic light scattering (DLS), and for the morphological feature using SEM, TEM etc. [38–42].

The subsequent proteomic analysis of the isolated exosomes from the cancer cells have reported to shed light in identification of potent exosomal biomarker in several cancer types such as breast, lung, liver, prostate, ovarian, colorectal cancer, glioblastoma etc., the specific exosomal proteins includes surface proteins, Rab family GTPases, annexins, flotullin, exosome biogenesis proteins such as, Alix, Tsg101 and ESCRT complex. Several other exosomal protein includes, Tetraspanins (CD63, CD9, CD81, CD53etc.), Hsp90, Hsp70, EpCam etc. can serve as an efficient exosomal markers. A study by Rupp et al. Has reported the exosomal CD24 could serve as an efficient circulating biomarker for the detection of breast cancer. Several other exosomal protein biomarkers reported in breast cancer diagnosis are EDIL3 and fibronectin, for early breast cancer detection using ELISA, which can also serve as treatment response marker as the level of these two tremendously decreased after surgery. Likewise the expression of Survivin, a apoptosis inhibitor was found to be higher in prostate cancer exosomes compared to the normal. The urinary exosomes also do possess significant amount of biomarker such as PCA3, TMPRSS2: ERG in prostate cancer [38, 43–46].

Several recent studies have reported the development of numerous exosome-based diagnostic platform viz.... Exochip, which is a microfluidic device developed by Kanwar and team based on the exosomal tetraspanin protein CD63 where, the exosomes gets bound with CD63 antibody and a fluorescent reporter using which, the exosomes can be quantified. Another type of analytical technique used in exosome diagnostics is ExoScreen, the technique developed by Yoshioka et al., that utilizes CD9 and CD147 antibodies alongside photosensitizing beads. Apart from the above two, yet another exosome diagnosing tool was developed by Zhao and team called Exosearch chip which is a comparatively simpler technique which enables the quantitative isolation of exosomes by the virtue of immunomagnetic beads. The Exosearch technique was successfully used for the quantification of ovarian cancer exosomes using the exosomal markers CA-125, EpCam and CD24 [38, 47–49].

Apart from exosomal proteins, the use of techniques such as RNA-sequencing and DNA sequencing for the analysis of genomic data of cancer-derived exosomes have shed light on yet another exosomal component as an efficient biomarker use i. e., nucleic acid especially the ncRNAs including miRNAs and lncRNA. The exosomal miRNA are considered a most appropriate exosomal biomarker as they are quite stable against Rnase dependent degradation. Till date numerous exosomal miRNAs have been characterized as a potent tumor marker in several cancer conditions to name a few are, miR-21, miR-141, miR-220a, miR-200b, miR-203, miR-205, miR-214 in case of ovarian cancer as reported by Taylor et al. Other examples for exosomal miRNA biomarkers includes miR-31, miR-196a, miR-1246, miR-191, miR-451a, miR-483-3p, miR-16a etc. in case of pancreatic cancer have also been reported by several studies. Other ncRNAs accounting for exosomal long noncoding RNA, circular RNA have also been reported pertaining to the development of exosomal biomarkers for early cancer detection [38, 50–52].

4. CRISPR in diagnosis

Along with its extensive use in the field of genome editing, CRISPR/Cas system has expanded its wings towards diagnostics where it is mainly involved in detection of specific nucleic acid such as genomic DNA, non-genomic DNA, RNA, and pathogenic microbe genomes. This could have been accomplished due to their natural ability of efficient nucleic acid recognition and editing, have been demonstrated to be extraordinary tools for specific nucleic acid detection. The CRISPR diagnostics are reported to have influence the targeting efficiency of the CRISPR guide RNA either in the presence or absence of nucleic acid cleaving potential of the Cas nucleases. The basic principle of CRISPR based diagnostics is, here the CRISPR/Cas components are modified in such a way that, they will emit the color or fluorescence with response to their binding with the target nucleic acid sequence in certain pathological conditions. Based on the involvement of specific effector protein, several CRISPR based diagnostic tools kits have been developed which are mainly belonging to class II of CRISPR/Cas classification viz.... dCas9, SHERLOCK, SHERLOCK v2, DETECTR, HOLMES for an efficient detection of pathological conditions. A brief characterization, mode of action, application and advancement of these CRISPR tool kits in general and with respect to cancer will be discussed in this section.

Being the first effector protein to be characterized, Cas9 not only plays a vital role in genome editing, rather with minor modifications such as in dCas9, which is a nuclease-deactivated Cas9 (also termed as dead Cas9), it is also reported to be involved in nucleic acid detection as a simple and programmed detection tool. The dCas9 system is designed with modification in the basic activity of the conventional Cas9 protein in terms of deactivating the nucleic acid cleavage potential and only retaining the specific binding ability to target dsDNA. This was accomplished by inducing two point mutations H840A and D10A in the HNH and RuvC nuclease domain of the conventional Cas9 effector protein [36, 53].

Alongside classy Cas9, three more novel class 2 effector Cas proteins Cas12a (prior referred as Cpf1), Cas13a (prior referred as C2c2)and Cas14a have been showed to have more potent diagnostic properties and have become the latest interest of the scientific community. Unlike the Cas9 nuclease, the latter mentioned effector proteins have the property of "Collateral cleavage", the property in which they can induce cleavage of the nearby sequence, which is not complementary to the designed crRNA upon detection and binding to the target nucleic acid sequence. Precisely, the when crRNA along with its effector protein either Cas12a or Cas13a recognize and bind to their target nucleic acid sequence either DNA or RNA

followed by their cleavage the activated effector protein also cleaves the nearby non targeted RNAs which does not emit the fluorescence until it is cleaved. With this, they offer a simple, fast, portable and reliable quantitative detection in diagnostics. It is this property of Cas12a and Cas13a, which has enabled effective tracing and detection of specific nucleic acid sequence, where the fluorescent ssDNA/ssRNA reporters are cleaved as a result of collateral cleavage.

Cas12a, which is previously referred to as Cpf1, are the variant of Cas effector protein which are RNA-guided, DNA-targeting enzyme, involved in type V of CRISPR/Cas classification. Unlike Cas9, these are reported to act or detect, bind and cleaves ssDNA. In contrast to this, Cas13a, which is previously referred to as C2c2 are RNA-guided targeting enzymes involved in type VI of CRISPR/Cas system and are specific for ssRNA. Based on the promiscuous Rnase ability of collateral cleavage of Cas12a as well as Cas13a several molecular diagnostic platforms have been developed in the recent years details of which will be discussed further [53–57].

As said before the collateral cleavage ability of Cas13a nuclease, lead to the development of a versatile *in vitro* nucleic acid detection platform named Specific High sensitivity Enzymatic Reporter UnLocking or simply abbreviated as SHERLOCK by Feng Zhang et al. in the year 2018, through technique of isothermal amplification along with recombinase polymerase amplification and T7 transcription, which has a wide spread application detecting viruses, pathogenic bacteria and in identifying tumor DNA mutation. Recent studies have reported the use of SHERLOCK in detecting two mutants BRAF V600E and EGFR L858R. An improved version of SHERLOCK, named SHERLOCKv2, was also came into existence with the ability to detect four viruses at the same time. Yet another CRISPR/Cas system involving Cas12a is also been found to be the basis of developing two more diagnostic tool (**Table 1**), named DETECTR (DNA endonuclease targeted CRISPR trans reporter) by Doudna et al. in 2018. The former has been found instrumental in diagnosing HPV + ve cervical cancer subtypes HPV 16 and HPV18 in both cell lines as well as clinical samples from the patients [53]. Apart from the above another diagnostic platform based on Cas12a has be developed called, one-hour low-cost multipurpose highly efficient system or simply HOLMES in the same year. Further experimentation focusing on the use of other Cas enzymes, Cas14 as well as CasX, which are Cas variants of type V CRISPR/Cas system alongside Cas12a. Development of newer diagnostic platform using these exceptionally smaller and compact RNA – guided nucleases made of 400-700 amino acids, targeting ssDNA are under progression for exploring their diagnostic potential as Cas14-DETECTR, yet another tool by Doudna and team [58, 59].

FEATURES	dCas9	SHERLOCK	DETECTR
Characteristics	cancer therapy by modifying DNA of target genes, stimulate tumor suppressor genes, knockdown oncogenes and tumor resistance pathways for targeted therapy	efficient, robust method to detect RNA and DNA, quick detection of infectious disease and involved in sensitive genotyping	genome editing tool based on its ability to stimulate genetic alteration in cells at sites of double-stranded DNA cut
Type of Cancer	Breast cancer, prostate cancer	Breast cancer	Cervical cancer
Target gene	AKT	EGFR L858R and BRAF V600E	HPV16, HPV18
References	[60]	[57, 61, 62]	[53, 59, 63]

Table 1.
Different CRISPR tools used for diagnosis of cancer malignancy.

5. Future of Exo CRISPR diagnostics

The two most important diagnostic markers Exosomes as well as CRISPR/Cas system and their features and advantages have been discussed thoroughly in the above sections. As mentioned before there are several exosome based diagnostic tools (ExoChip, ExoScreen, Exosearch) as well as CRISPR/Cas based diagnostic platforms (SHERLOCK, SHERLOCKv2, DETECTR and HOLMES) for the efficient diagnosis of the pathological conditions. Even though both of the fore said diagnostic platforms offers a greater advantage towards cancer diagnostics, these are not devoid of the cons pertaining to the exosomal detection, these are said to be in requirement of sophisticated sensing methodologies involving expensive equipments, and kits. On the other hand the CRISPR/Cas system, are in requirement of a efficient delivery in order to minimize the degradation by the systemic enzymes. With this insight, we hereby, suggest the use of a combinational technique made of both Exosomes and CRISPR where one can circumvent the drawback of other and becoming a full-fledged diagnostic platform.

In order to achieving efficient delivery of CRISPR/Cas system towards its target is an important step in the process of diagnostics and hence developing novel delivery system with higher efficiency and low immunogenicity and cytotoxicity is essential for the diagnostic applications. Off target effect of CRISPR system might lead to false acquisition of data for getting rid of which, the system has to be properly directed towards the target cell to achieve accuracy and efficiency. Different types of delivery system are available in present days this includes Adeno-associated viruses, Adenoviral vector, Lentiviral vector, Microinjection, electrophoresis, Lipid nanoparticle, cell penetrating peptides mediated and Gold nano particle mediated approaches [64]. Exosome, are reported to act as a promising carrier for CRISPR delivery, have an advantage over the other delivery system due to their natural biocompatible characteristics, high stability, low immunogenicity, and long circulation. Some exosomes can even have a high capacity to escape from degradation or clearance by the immune system [64, 65].

Recent advancement on this combinational approach has been reported by Yi He et al., and team where they have constructed a combinational tool called Aptamer-RPA-TMA-Cas13a Assay (ARTCA), a CRISPR/Cas13 based platform with modification for a significant detection of exosomal PD-L1 i. e., programmed cell death receptor, a promising biomarker for cancer immunotherapy monitoring, directly from the serum. This was accomplished by using an PD-L1 specific DNA aptamer which is further amplified by the aid of recombinase polymerase amplification (RPA) which is intern coupled with TMA (transcription-mediated amplification). By the aid of this tremendous diagnostic tool the expression level of PD-L1 in circulating tumor exosomes was constructed as reliable biomarker detection system. The same team have also reported the use of Cas12a for the construction of yet another CRISPR/Cas strategy, termed the apta-HCR-CRISPR assay, in order to detect nucleolin+ ve and PD-L1 + ve tumor derived exosomes [66–68]. With this we hereby, summarize the use of Exosome/CRISPR/Cas combo, where the exosomes can be effectively used for the delivery of CRISPR targeting the detection of specific nucleic acid or the array where the CRISPR/Cas system can be efficiently be targeted for the detection of exosomal biomarkers enlisted in the prior sections for early detection of cancer. The precise mechanism of Exosome/CRISPR/Cas system has been depicted in **Figure 3** where, the fore said combination can be used for both *In vivo* as well as *In vitro* diagnosis of tumor as picturized in **Figure 3A**. Here the exosomes derived from different sources can be used as a effective vehicle for site specific delivery of CRISPR/Cas detection system to avoid off target effect and false acquisitions. As depicted in **Figure 3B**, which is yet another possibility of using this

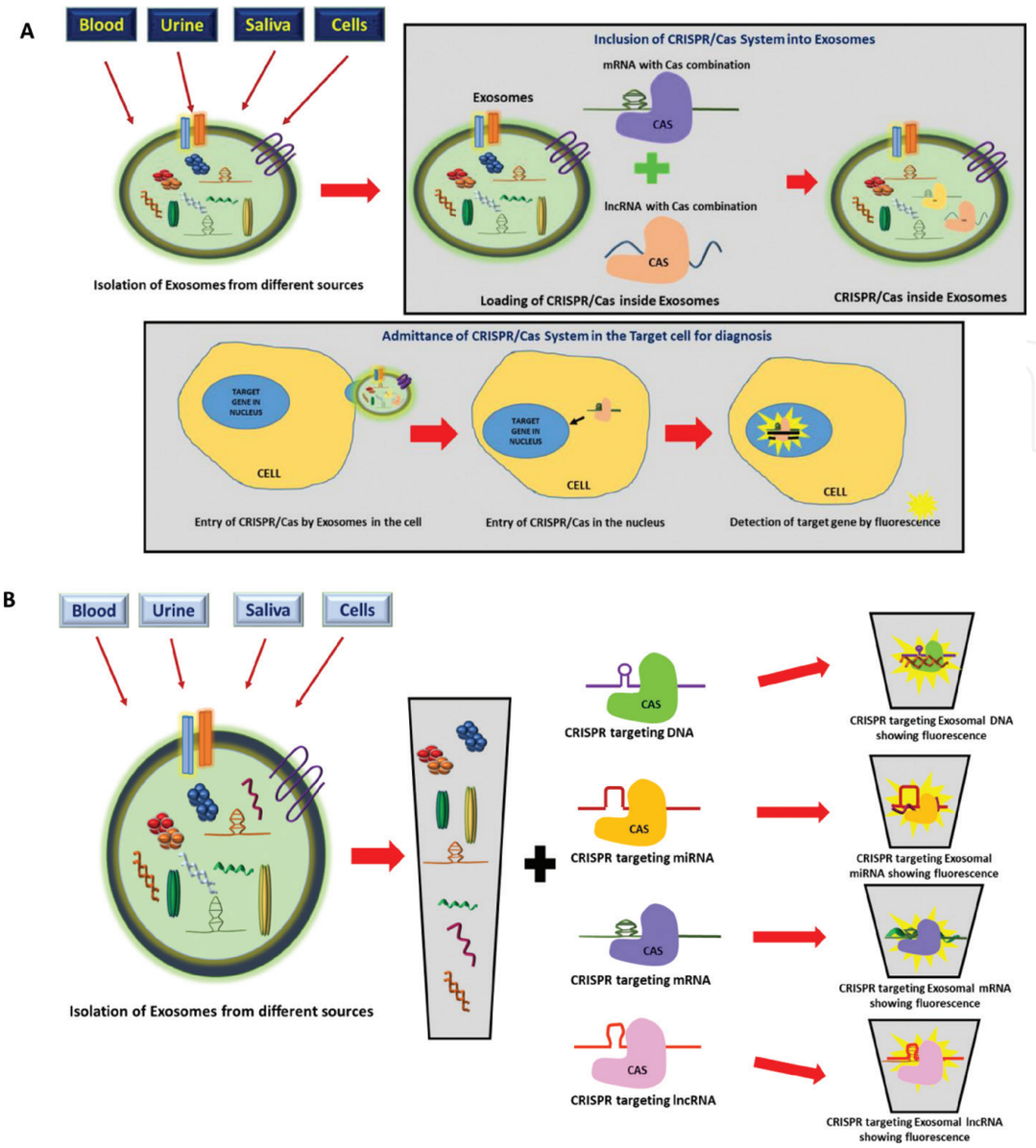

Figure 3.
Schematic representation of mechanism of action of exosome/CRISPR/Cas system for efficient cancer diagnosis. A) Exosome mediated delivery of CRISPR/Cas for effective detection of cancer. B) CRISPR/Cas based detection of exosomal biomarkers for early cancer diagnosis.

significant combination which can be optimized for *Ex Vivo* detection of cancer exosomal biomarkers that are secreted in early stages of cancer progression such as exosomal DNA, exosomal coding and noncoding RNAs (miRNA, lncRNA) and exosomal proteins. This can be beneficial for developing Exo CRISPR based early cancer detection kits.

6. Conclusion

It is generally stated, "The sooner, the better" which is more appropriate when it comes to diagnostics. If any pathological condition is detected in its earlier phases, the options for the effective treatment of that particular disease will be a lot more efficient than that of in the later phases. As mentioned in the fore said introduction, there are several diagnostic techniques available for the detection of cancer. Though these techniques offer a greater aid in the diagnosis of several pathological conditions including cancer, these are not devoid of the disadvantages in being pricey,

time consuming and they do pose the threat to cause infections leading to worsening of the condition. For instance, in case of biopsies, even though it is considered the golden standard in the cancer diagnosis, they do have the threat in causing infections as, it is an invasive method. The threat of repetitive exposure to the radiations such as x-rays might also effect otherwise along with being useful in diagnosis of solid tumors.

As mentioned before the greatest drawback of the conventional diagnostic tools when it comes to cancer is, most of these techniques can only detect the disease in its later stages. Such as, in case of laboratory techniques, which uses of blood and urine for the presence of conventional biomarkers enlisted above, can be detected in the later stages when cancer has already become metastatic as these biomarker enters the blood stream in the later stages. This is where the role of exosomes comes into play as an efficient biomarker for early cancer detection as, there are several reports stating that the exosomes from the cancer cells are secreted more compared to the exosomes secreted from the normal cells, in order to aid in further spreading of the disease to the distant sites of the body. Moreover, these exosomes have unleashed the site for lesser - noninvasive method of diagnosis as it is present in all the body fluids enlisted in the introduction section.

Several research reports supports the use of CRISPR/Cas system for its substantial role in diagnosis. In recent years both exosomes as well as CRISPR/Cas per se has proven to be an excellent diagnostic aid. The CRISPR based diagnostics have unmatched advantages over the conventional diagnostic tools, and aided the researchers with its precision targeting efficiency, high specificity and single base specificity enabling early screening and detection of cancer susceptible genes and sensitivity towards the target nucleic acid and with its low time consuming and monetary costs. Here in this book chapter, we have summarized the possible combinatorial effect of these two tools, which might offer an additional competence. Detection of cancer in its early stages might be handy in improving the efficiency of its treatment and might reduce the possibility of cancer relapse as the recurrence rate is reported to be comparatively more in the later stages of cancer making it incurable.

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References

[1] Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*. 2021; 71(3):209-249. doi:10.3322/caac.21660.

[2] Zaorsky NG, Churilla TM, Egleston BL, et al. Causes of death among cancer patients. Ann Oncol. 2017;28(2):400-407. doi:10.1093/annonc/mdw604.

[3] Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. Proc Natl Acad Sci U S A. 1995;92(12):5258-5265. doi:10.1073/pnas.92.12.5258Hulvat MC. Cancer Incidence and Trends. Surg Clin North Am. 2020;100(3):469-481. doi:10.1016/j.suc.2020.01.002.

[4] Anand P, Kunnumakkara AB, Sundaram C, etal. Cancer is a preventable disease that require major lifestyle changes. *Pharm Res.* 2008; 25 (9): 2097-2116. doi: 10.1007/s11095-008-9661-9.

[5] Blackadar CB. Historical review of the causes of cancer. *World J Clin Oncol*. 2016; 7 (1): 54-86. doi:10.5306/wjco.v7.i1.54.

[6] Malik MA, Sepehri Z, Shah ZA. Cancer: disease caused by alteration of genes. *Int J Basic Sci Med.* 2016; 1 (2): 37-39. doi: 10.15171/ijbms.2016.09.

[7] Carbone A. Cancer classification at the crossroads. *Cancers.* 2020; 12 (4): 980. http://doi.org/10.3390/cancers12040980.

[8] Akalin, A., Mu, X., Kon, M. *et al.* Classification of malignant and benign tumors of the lung by infrared spectral histopathology (SHP). *Lab Invest* **95,** 406-421 (2015). https://doi.org/10.1038/labinvest.2015.1.

[9] Sobin LH. The international histological classification of tumors. *Bull World Health Organization*. 1981; 59(6): 813-819.

[10] Rosen RD, Sapra A. TNM Classification. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK553187/.

[11] Brierley JD, Gospodarowicz MK, Wittekind Ch., editors. International Union Against Cancer (UICC). TNM Classification of Malignant Tumours, 8th edn : Chichester, UK Wiley Blackwell 2017.

[12] Hortobagyi GN, Edge SB, Giuliano A. New and Important Changes in the TNM Staging System for Breast Cancer. Am Soc Clin Oncol Educ Book. 2018; 38: 457-467. doi:10.1200/EDBK_201313.

[13] Scheel BI, Holtedahl K. Symptoms, signs, and tests: The general practitioner's comprehensive approach towards a cancer diagnosis. *Scand J Prim Health Care.* 2015; 33 (3): 170-177. Doi: 10.3109/02813432.2015.1067512.

[14] Koo MM, Hamilton W, Walter FM, Rubin GP, Lyratzopoulos G. Symptom signatures and diagnostic timeliness in cancer patients: A review of current Evidence. Neoplasia. 2018; 20(2): 165-174. Doi: 10.1016/j.neo.2017.11.005.

[15] Nagpal M, Singh S, Singh P, Chauhan P, Zaidi MA, Tumor markers: A diagnostic tool. *Natl J Maxillofac surg.* 2016; 7 (1): 17-20. doi: 10.4103/0975-5950.196135.

[16] Henry NL, Hayes DF. Cancer biomarkers. *Mol Oncol.* 2012; 6 (2): 140-146. doi: 10.1016/j.molonc.2012.01.010.

[17] Ferguson JL, Turner SP. Bone Cancer: Diagnosis and Treatment Principles. Am Fam Physician. 2018;98(4):205-213.

[18] Pucci C, Martinelli C, Ciofani G. Innovative approaches for cancer treatment: current perspectives and new challenges. Ecancermedicalscience. 2019;13:961. doi:10.3332/ecancer.2019.961.

[19] Chuanjiang He, Shu Zheng, Yan Luo and Ben Wang. Exosome Theranostics: Biology and Translational Medicine. *Theranostics*. 2018; 8(1): 237-255. doi: 10.7150/thno.21945.

[20] Doyle LM, Wang MZ. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cell*. 2019; 8 (7): 727. doi: 10.3390/cells8070727.

[21] Harding CV, Heuser JE, Stahl PD. Exosomes: looking back three decades and into the future. *J Cell Biol*. 2013; 200 (4):367-371. doi: 10.1083/jcb.201212113.

[22] Harding C, Stahl P. Transferrin recycling in reticulocytes: pH and iron are important determinants of ligand binding and processing. *Biochem Biophys Res Commun*. 1983; 113 (2): 650-658. doi: 10.1016/006-291x(83)91776-x.

[23] Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*. 1983; 33 (3): 967-978. doi: 10.1016/0092-8674(83)90040-5.

[24] Gurunathan S, Kang MH, Kim JH. A Comprehensive Review on Factors Influences Biogenesis, Functions, Therapeutic and Clinical Implications of Exosomes. Int J Nanomedicine. 2021;16:1281-1312. Published 2021 Feb 17. doi:10.2147/IJN.S291956.

[25] Willms, E., Johansson, H., Mäger, I. et al. Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci Rep 6, 22519 (2016). https://doi.org/10.1038/srep22519.

[26] Huda MN, Nafiujjaman M, Deaguero IG, et al. Potential Use of Exosomes as Diagnostic Biomarkers and in Targeted Drug Delivery: Progress in Clinical and Preclinical Applications. ACS Biomater Sci Eng. 2021;7(6):2106-2149. doi:10.1021/acsbiomaterials.1c00217.

[27] Zhang Y, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci*. 2019;9:19. Published 2019 Feb 15. doi:10.1186/s13578-019-0282-2.

[28] Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science*. 2020; 367(6478):eaau6977. doi:10.1126/science.aau6977.

[29] Hille F, Charpentier E. CRISPR-Cas: biology, mechanisms and relevance. Phil. Trans. R. Soc. B. 2016; 371: 20150496. doi:10.1098/rstb.2015.0496

[30] Makarova, K., Wolf, Y., Alkhnbashi, O. et al. An updated evolutionary classification of CRISPR–Cas systems. *Nat Rev Microbiol*. 2015;13, 722-736. doi:10.1038/nrmicro3569

[31] Makarova KS, Haft DH, Barrangou R, et al. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol*. 2011;9(6):467-477. doi:10.1038/nrmicro2577

[32] Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. 2014;157(6):1262-1278. doi:10.1016/j.cell.2014.05.010

[33] Rajat M. Gupta and Kiran Musunuru. Expanding the genetic editing tool kit: ZFNs, TALENs, and

CRISPR-Cas9. Review: *J Clin Invest*. 2014; 124(10):4154-4161. doi:10.1172/JCI72992

[34] Hiroko Koike-Yusa, Yilong Li, E-Pien Tan, Martin Del Castillo Velasco-Herrera & Kosuke Yusa. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol*. 2014;32(3):267-273. doi:10.1038/nbt.2800

[35] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012; 337 (6096): 816-821. doi: 10.1126/science.1225829

[36] Liu Z, Dong H, Cui Y, Cong L, Zhang D. Application of different types of CRISPR/Cas-based systems in bacteria. Microb Cell Fact. 2020; 19 (1): 172. doi: 10.1186/s12934-020-01431-z.

[37] Zhang W, Peng P, Shen K. Role of Exosomal Shuttle RNA in Cell-to-cell Communication. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao*. 2016; 38 (4): 480-483. doi: 10.3881/j.issn.1000-503X.2016.04.020.

[38] Soung YH, Ford S, Zhang V, Chung J. Exosomes in Cancer Diagnostics. Cancers (Basel). 2017;9(1):8. Published 2017 Jan 12. doi:10.3390/cancers9010008.

[39] Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics.* 2017; 7(3): 789-804. doi: 10.7150/thno.18133.

[40] Yu LL, Zhu J, Liu JX, Jiang F, Ni WK, Qu LS, Ni RZ, Lu CH, Xiao MB. A comparison of Traditional and Novel Method for the separation of Exosomes from Human Samples. *BioMed Research International*. 2018: 2018:36364563. doi: 10.1155/2018/3634563.

[41] Patel GK, Khan MA, Zubair H, Srivastava SK, Khusman M, Singh S, Singh AP. Comparative analysis of exosomes isolation methods using culture supernatant for optimum yield, purity and downstream applications. *Nat Sci Rep*. 2019; 9(1): 5335. doi: 10.1038/s41598-019-41800-2.

[42] Lobb RJ, Becker M, Wen SW, Wong CSF, Wiegmans AP, Leimburger A, Moller A. Optimized exosome isolation protocol for cell culture supernatant and human plasma. *Journal of Extracellular Vesicles*. 2015; 4(1): 27031. doi: 10.3402/jev.v4.27031.

[43] Rupp AK, Rupp C, Keller S, et al. Loss of EpCAM expression in breast cancer derived serum exosomes: role of proteolytic cleavage. Gynecol Oncol. 2011;122(2):437-446. doi:10.1016/j.ygyno.2011.04.035.

[44] Moon PG, Lee JE, Cho YE, et al. Identification of Developmental Endothelial Locus-1 on Circulating Extracellular Vesicles as a Novel Biomarker for Early Breast Cancer Detection. Clin Cancer Res. 2016;22(7): 1757-1766. doi:10.1158/1078-0432.CCR-15-0654.

[45] Khan S, Jutzy JM, Valenzuela MM, et al. Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer. PLoS One. 2012;7(10):e46737. doi:10.1371/journal.pone.0046737.

[46] Nilsson J, Skog J, Nordstrand A, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Br J Cancer. 2009;100(10):1603-1607. doi:10.1038/sj.bjc.6605058.

[47] Kanwar SS, Dunlay CJ, Simeone DM, Nagrath S. Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes. Lab Chip. 2014;14(11):1891-1900. doi:10.1039/c4lc00136b.

[48] Yoshioka Y, Kosaka N, Konishi Y, et al. Ultra-sensitive liquid biopsy of

circulating extracellular vesicles using ExoScreen. Nat Commun. 2014;5:3591. Published 2014 Apr 7. doi:10.1038/ncomms4591.

[49] Zhao Z, Yang Y, Zeng Y, He M. A microfluidic ExoSearch chip for multiplexed exosome detection towards blood-based ovarian cancer diagnosis. Lab Chip. 2016;16(3):489-496. doi:10.1039/c5lc01117e.

[50] Huang T, Deng CX. Current Progresses of Exosomes as Cancer Diagnostic and Prognostic Biomarkers. Int J Biol Sci. 2019;15(1):1-11. Published 2019 Jan 6. doi:10.7150/ijbs.27796.

[51] Zhou, B., Xu, K., Zheng, X. et al. Application of exosomes as liquid biopsy in clinical diagnosis. Sig Transduct Target Ther 5, 144 (2020). https://doi.org/10.1038/s41392-020-00258-9.

[52] Ariston Gabriel, A.N., Wang, F., Jiao, Q. et al. The involvement of exosomes in the diagnosis and treatment of pancreatic cancer. Mol Cancer 19, 132 (2020). https://doi.org/10.1186/s12943-020-01245-y.

[53] Zhu CS, Liu CY, Qiu XY, et al. Novel nucleic acid detection strategies based on CRISPR-Cas systems: From construction to application. *Biotechnol Bioeng*. 2020; 117(7): 2279-2294. doi:10.1002/bit.27334.

[54] Xu Y, Li Z. CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. *Comput Struct Biotechnol J*. 2020;18:2401-2415. Published 2020 Sep 8. doi:10.1016/j.csbj.2020.08.031.

[55] Yao R, Liu D, Jia X, Zheng Y, Liu W, Xiao Y. CRISPR-Cas9/Cas12a biotechnology and application in bacteria [published correction appears in Synth Syst Biotechnol. 2020 Oct 12; 5(4): 328. *Synth Syst Biotechnol*. 2018; 3 (3): 135-149. Published 2018 Oct 3. doi:10.1016/j.synbio.2018.09.004.

[56] Jolany vangah S, Katalani C, Boone HA etal. CRISPR-Based Diagnosis of Infectious and Noninfectious Diseases. [published correction appears in *Biol Proced Online*. 2020 Nov 7;22(1):24]. *Biol Proced online.* 2020; 22; 22. Published 2020 sep 14. https://doi.org/10.1186/s12575-020-00135-3.

[57] Abudayyeh OO, Gootenberg JS, Essletzbichler P etal. RNA targeting with CRISPR-Cas13. *Nature.* 2017; 550 (7675): 280-284. doi: 10.1038/nature24049.

[58] Savage DF. Cas14: Big Advances from Small CRISPR Proteins. *Biochemistry.* 2019; 58(8): 1024-1025. http://doi.org/10.1021/acs.biochem.9b00035.

[59] Harrington LB, Brustein D, Chen JS, Paez-Espino D, Ma E, Witte IP, Cofsky JC, Kyrpides NC, Banfield JF and Doudna JA. Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. *Science.* 2018; 362(6416): 839-842. doi: 10.1126/science.aav4294.

[60] Martinez-Escobar A, Luna-Callejas B, Ramón-Gallegos E. CRISPR-dCas9-Based Artificial Transcription Factors to Improve Efficacy of Cancer Treatment With Drug Repurposing: Proposal for Future Research. *Front Oncol*. 2021;10:604948. Published 2021 Feb 3. doi:10.3389/fonc.2020.604948.

[61] Mintz RL, Gao MA, Lo K, Lao YH, Li M, Leong KW. CRISPR Technology for Breast Cancer: Diagnostics, Modeling, and Therapy. Adv Biosyst. 2018;2(11):1800132. doi:10.1002/adbi.201800132.

[62] Gootenberg JS, Abudayyeh OO, Lee JW, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science.

2017;356(6336):438-442. doi:10.1126/science.aam9321.

[63] Chen JS, Ma E, Harrington LB, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity [published correction appears in Science. 2021 Feb 19;371(6531):]. *Science*. 2018;360(6387):436-439. doi:10.1126/science.aar6245.

[64] Zhang S, Shen J, Li D, Cheng Y. Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. Theranostics. 2021;11(2):614-648. Published 2021 Jan 1. doi:10.7150/thno.47007

[65] Yang B, Chen Y, Shi J. Exosome Biochemistry and Advanced Nanotechnology for Next-Generation Theranostic Platforms. Adv Mater. 2019;31(2):e1802896. doi:10.1002/adma.201802896

[66] Y. He, Y. Wu, Y. Wang, X. Wang, S. Xing, H. Li, S. Guo, X. Yu, S. Dai, G. Zhang, Applying CRISPR/Cas13 to Construct Exosomal PD-L1 Ultrasensitive Biosensors for Dynamic Monitoring of Tumor Progression in Immunotherapy, Adv. Therapeut. 2020: 3 (10); https://doi.org/10.1002/adtp.202000093.

[67] Xing S, Lu Z, Huang Q, et al. An ultrasensitive hybridization chain reaction-amplified CRISPR-Cas12a aptasensor for extracellular vesicle surface protein quantification. Theranostics. 2020;10(22):10262-10273. Published 2020 Aug 13. doi:10.7150/thno.49047.

[68] De Jong, O.G., Murphy, D.E., Mäger, I. et al. A CRISPR-Cas9-based reporter system for single-cell detection of extracellular vesicle-mediated functional transfer of RNA. Nat Commun 11, 1113 (2020). https://doi.org/10.1038/s41467-020-14977-8.