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Written in Blood: Liquid Biopsies for Cervical Cancer Screening

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BACKGROUND INFORMATION

Cervical cancer (CC) is one of the most prevalent cancers in the world, causing over 273,000 deaths globally per year [7,8]. The disease ranks second in cancer mortality for developing countries despite of advancements in prevention and screening [8]. Virtually all CC cases are caused by chronic Human papillomavirus (HPV) infection. HPV can be both sexually and vertically transmitted. Recent studies found that children born to HPV-positive mothers are five times more likely to get HPV [9].

HPV belongs to a family of ubiquitous DNA viruses that infects human epithelial cells and includes over 200 types [7]. HPV is grouped into low-risk and high-risk HPV. Low-risk HPV types, such as 6 and 11, are associated with benign cutaneous warts that resolve naturally within two years [10]. High-risk HPV types, such as 16 and 18, are associated with expression of oncogenes and suppression of tumor suppression genes [10]. Persistent infection with high-risk HPV can lead to precancerous lesions and eventually cancer if left untreated. See Figure 1 for an illustration of HPV infection progression to CC. So, CC prevention programs, such as vaccination (Gardasil® and Cervarix®) and annual screening (Pap and HPV testing), focus on protection and detection of those high-risk HPV types [7].

Recently, a review of CC screening in 57 countries found that only 18% of women in developing countries had a Pap test during the last 3 years, compared to 63% of women in developed countries [11]. It was proposed that for developing countries, the nature of those tests hinder the implementation of CC screening and participation among women. Pap testing is a form of invasive biopsy that involves the extraction and visual detection of abnormal cervical cells. In addition, Pap testing is only offered to women ages 20 years and older due to sampling limitations [11]. Pap testing is also inaccurate, and has a 20-30% false positive rate [12]. Thus, this test requires women to be re-tested every 1 to 3 years. HPV testing is another common CC screening test offered to women ages 30 years and older [12]. This test is also invasive and requires clinical tests, most commonly *in-situ* hybridization, to detect HPV DNA in the host cell [12]. As implicated, current CC screening

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programs require expensive equipment to extract and preserve samples, training to perform the tests, and annual screening participation. These pose significant challenges for low-resource nations and indicate a growing demand for alternative CC diagnostic methods.

A novel type of diagnostics, known as liquid biopsy, has the potential to replace existing screening techniques. Liquid biopsy is a non-invasive method used to collect and analyze the molecular content of biofluids [13]. Most commonly used biofluid is blood, but others, such as urine, are also used for liquid biopsy [13]. The goals of this technology are to screen for disease-associated genetic abnormalities and to monitor disease progression in affected patients by using sequencing techniques. In 2016, the FDA approved the world's first liquid biopsy companion diagnostic for non-small cell lung cancer (Coba EGFR Mutation Test v2) [14]. This liquid biopsy detects individuals with specific EGFR mutations to be eligible for treatment with a novel tyrosine inhibitor. Since then, there has been a push to apply liquid biopsy technologies in other diseases. The non-invasive nature of the test allows for multiple sampling and generates results at higher accuracy compared to current screening methods. Specific to CC, liquid biopsies can also improve women's admittance to cervical screening programs in developing countries. This article aims to address the enormous potential of liquid biopsies as a screen for CC and focuses on how liquid biopsies can be applied in low-resource nations.

> "Cervical cancer (CC) is one of the most prevalent cancers in the world, causing over 273,000 deaths globally per year"

RESEARCH QUESTIONS

The holy grail of cancer research is to create an effective, low-cost diagnostics tool that generates highly accurate results and allows for early detection. Liquid biopsy provides a non-invasive, real-time detection alternative to traditional cervical screening approaches. The clinical implementation of liquid biopsy for cervical screening can greatly enhance patient outcomes and enable more targeted, personalized treatments. In addition, future liquid biopsy developments to speed and accessibility can lead the way to point-of-care testing. The next-generation testing can rapidly assess the patient at the bedside and generate results on the spot. This can greatly reduce patient mortality as clinicians can intervene with treatments that halt disease progression.

Three important questions must be explored to investigate the potential of liquid biopsies for cervical screening. The first question addresses the mechanisms of liquid biopsy and how it can be used specifically for CC detection. After elucidating the key aspects of liquid biopsy and its applications, it is important to examine the various CC biomarkers that can be used for liquid biopsy. A special emphasis will be placed on exosome-based liquid biopsy due to its applicability in developing countries. As an extension into those countries, the next question addresses the different socio-economic considerations for patient screening in those areas. This is important to understand as the implementation of novel diagnostics may be affected by patient attitudes and the nature of screening programs in developing countries.

PROJECT NARRATIVE

How are liquid biopsies used for CC detection and monitoring?

The field of liquid biopsy is currently divided to three main approaches: circulating tumor cells (CTCs), cell-free DNA (cfDNA), and exosomes. These approaches vary in clinical relevance and biological implications. The following provides an overview of the three

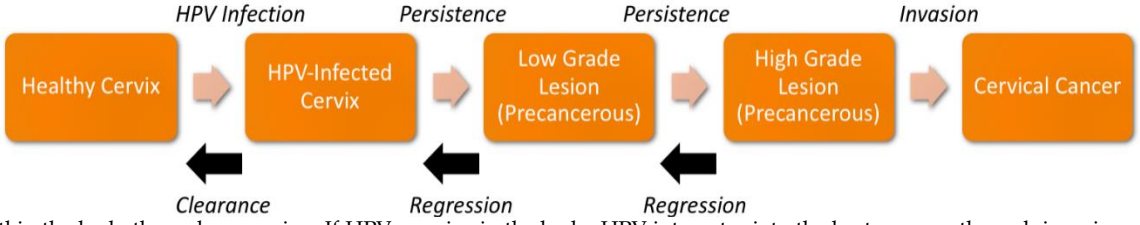


FIG. 1 Pro persisting HPV infec PV can be cleared within the body through regression. If HPV remains in the body, HPV integrates into the host genome through invasion, and causes CC.

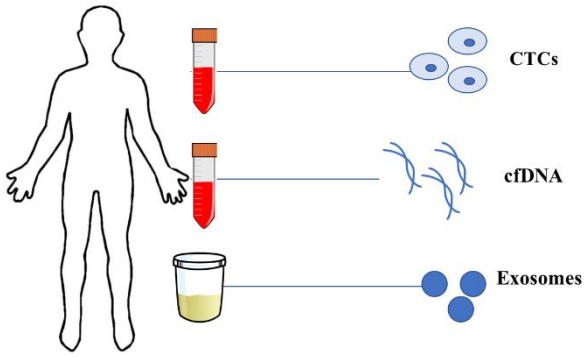
approaches and investigates their applicability to cervical screening.

CTCs are intact cells that are shed from the primary tumor and act as seeds for secondary tumors [15]. Several reports have showed a strong correlation between CTC counts and non-cervical cancer progression in patients, suggesting an essential role of CTCs in metastasis [16]. Using cell isolation technologies, CTCs are isolated and molecularly characterised using cytometric approaches, *in-situ* hybridization (FISH), and RNA/DNA isolation [16]. Characterizing CTCs yields a range of important genetic information, such as RNA/DNA mutations and cancer biomarkers, that can predict patient survival.

cfDNA are fragmented pieces of DNA found in the plasma [15]. The entry of cfDNA into the plasma is believed to be from cell apoptosis. Although cfDNA is also associated with non-malignant cells, analyzing the genetic content of cfDNA can reveal key mutations that are responsible for disease [15]. Quantifying cfDNA can also detect cancer stage. There has been research on the use of cfDNA for non-cervical cancers. One land-mark study by Diehl *et al.* found a positive correlation between cfDNA levels with advanced colorectal cancer [17]. The authors employed a combination of real-time PCR and BEAMing to quantify and characterize cfDNA. Using these techniques, they found that patients with worsening cancer had elevated levels of mutant cfDNA compared to patients with earlier stage cancers [17].

Exosome field has grown exponentially over the years, and are implicated in liquid biopsy. Studies have shown exosomes as important cargo-carrying vesicles of RNA, DNA, and protein, and function in cell-cell communication [15]. There has been some research showing the use of exosome-based liquid biopsy for CC detection. Zhang *et al.* found exosomes containing long noncoding RNA (lnRNA) in cervicovaginal lavage of CC patients [13]. Cervicovaginal lavage is fluid collected from the cervix and vagina after flushing with PBS. Using qRT-PCR, authors detected lncRNA MALAT1 and HOTAIR that are associated with oncogene regulation [18]. Additionally, the expression levels of lncRNAs were also elevated in HPV-positive patients compared to healthy volunteers [18]. See Figure 2 for an illustration of the different liquid biopsy sources.

In theory, combining the three liquid biopsy approaches enables detection of CC stages. CTCs can be used for advanced stage CC patients to monitor cancer progression and to assess treatment efficacy. For instance, an elevation of CTCs informs clinicians of worsening disease and signals the need for more aggressive interventions, such as chemotherapy. cfDNA

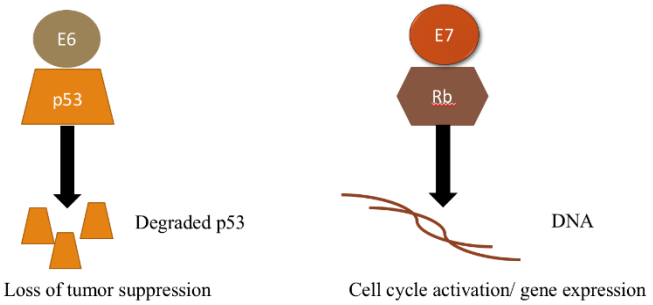


can identify key mutations that drive early CC development. Sequencing cfDNA in the plasma can identify hallmark mutations, such as tumor suppressor gene *TP53* and proto-oncogene *PIK3CA*, that are expressed early by CC cells [19]. Exosomes provide complete RNA transcription profiles to identify upregulation of oncogene expression during the initial stages of CC [16]. Clinicians can then choose targeted therapies that down-regulate those oncogenes, and thus stopping disease progression.

FIG. 2 Sources of liquid biopsy: Blood samples are used to detect CTCs, cfDNA, and exosomes. The use of other biofluids, such as urine, is exclusive to exosome-based liquid biopsy.

Although CTCs and cfDNA demonstrate enormous potential in CC screening, the key limitations are the stringent sample processing and storage requirements. Isolation of CTCs require processing of whole blood within 96 hours, so bio-banking is not feasible [15]. In addition, CTCs are highly fragile and quickly degrade in blood, so cold storage (-20^{0}C or -80^{o}C) is essential [20]. Similarly, cfDNA is easily degradable, with only a 15-minute half-life [20]. Exosomes, on the other hand, have shown to be stable under different storage conditions, including 4^{o}C [21]. This reduces the need for expensive freezers to store samples. Additionally, exosomes are found in all biofluids, from saliva to urine, whereas CTCs and cfDNA are restricted to blood [22]. This improves sampling accessibility and ease of collection. Due to these advantages, exosome-based liquid biopsy is most applicable for developing countries that lack adequate medical resources. The next question investigates the cervical cancer biomarkers for exosome-based liquid biopsies.

What are some cervical cancer biomarkers that can be used for exosome-based liquid biopsies?



E6/E7 HPV viral proteins are well-known mediators of cell immortalization by functionally inactivating p53 and retinoblastoma (Rb), which are two important tumor suppressor proteins [23]. See Figure 3 for E6/E7

FIG. 3 E6/E7 inhibition of p53 and Rb: E6 binds to p53 tumor suppressor at higher affinity, which results in the degradation of p53. This leads to loss of tumor suppression, and prevention of cell death. E7 binds to phosphorylated Rb. This results in pRb loss of function and deregulates the cell cycle. Damaged cells can keep growing and accumulate more mutations.

inhibition mechanism of p53 and Rb. Although E6/E7 genes are expressed highly in host cells and are used as biomarkers, recent studies have found low levels of E6/E7 in exosomes [24]. Research into biomarker discovery have now shifted their focus to two E6/E7-regulated molecules that are found more commonly in exosomes. These are exosomal oncogenic microRNA (miRNA) and survivin.

Exosomal oncogenic miRNAs are endogenous, non-protein coding small RNAs that are responsible for oncogene regulation [25]. Honegger *et al* proposed that exosomal miRNA concentration is dependent on E6/E7. Unlike other tumor viruses, such as Epstein-Barr Virus (EBV), HPV does not encode its own viral miRNA [26]. Instead, HPV E6/E7 upregulates cellular oncogenic miRNA production, which are then packed into exosomes. Using deep sequencing analysis, authors found that sustained *E6/E7* expression increased concentrations of miRNA 17~92 cluster, which repress anti-proliferation gene *p21* in HPV-positive cells [27]. Further qRT-PCR analysis validated E6/E7-mediated modulation of cellular miRNA. Authors also silenced E6/E7 via siRNA interference to determine the effects on miRNA production. As expected, E6/E7 repression was correlated with the reduction of several pro-tumorigenic miRNAs, which led to increased *p21* expression [27].

Survivin is a member of the inhibitor of apoptosis proteins (IAP) family that participates in tumor cell proliferation and survival [28]. Studies found survivin in exosomes of HPV-positive cells, fueling the potential of survivin as another promising biomarker [24]. Honegger *et al.* detected elevated levels of survivin in exosomes released from HeLa cells expressing E6/E7 [24]. It is proposed that E6 activity increases *survivin* expression through inhibition of p53. p53 is a known repressor of *survivin* and thus, the inhibition of p53 by E6 will upregulate *survivin* expression. Monitoring survivin concentrations in the exosome have profound implications for CC detection and treatment. Specifically, tracking survivin can predict response to chemotherapy. Research have shown that survivin reduces tumor cell death by increasing their resistance against common anticancer agents, such as paclitaxel and cisplatin [29].

The use of exosomes to detect biomarkers can have several impacts on CC diagnosis. As previously mentioned, exosomes are found in a variety of biofluids. This can diagnose HPV much earlier than current diagnostic approaches. In fact, HPV infection can be detected in the developing fetus using exosomes from amniotic fluid [9]. This is promising for HPV-positive pregnant women, as clinicians can analyze the exosomal content during routine amniocentesis to determine if HPV has passed onto the child. For HPV-infected patients, monitoring exosomal oncogenic miRNA can predict disease progression and allow clinicians to intervene with more potent therapies if miRNA levels rise. Patients with exosomal survivin may be prescribed survivin inhibitors, such as withanone and YM155, to improve their response to chemotherapy [30].

The extraction of exosomes from easily accessible biofluids remains the main advantage compared to cfDNA and CTC. However, the approach requires the use of expensive exosome isolation kits and detection instruments. Low-resource nations might be reluctant to spend millions on such novel technology, and combined with a variety of socio-economic barriers, it can be challenging to implement liquid biopsy in those nations. The following evaluates the bioethical implications of cervical screening in developing countries.

What are some socio-economic considerations for patient screening in developing countries?

Presently, few developing countries can implement effective cervical screening programs. An effective screening program should ensure adequate monitoring and follow-up of patients, especially for women who are HPV-positive and engage in high-risk behavior, such as smoking. The program should also be held on-site, with suitable equipment and access to trained personnel [31]. For instance, Pap testing requires specialized biopsy instruments to extract cervical cells,

storage equipment to store samples, and microscopes to look for abnormal cells. For many developing countries, lack of equipment and specialized training greatly affect sensitivity of Pap testing. In India, Pap testing sensitivity is only 62% compared to the US where the sensitivity is 87% [32,33]. The sensitivity of cervical screening drops further to 20% when unaided visual inspection (visually inspecting the cervix) is implemented in rural-India [34]. This becomes especially problematic for women who are screened only once in their lifetimes. HPV testing offers greater sensitivity, but costs around $20 to $30 (US dollars) per test, which is expensive for low-resource nations [33].

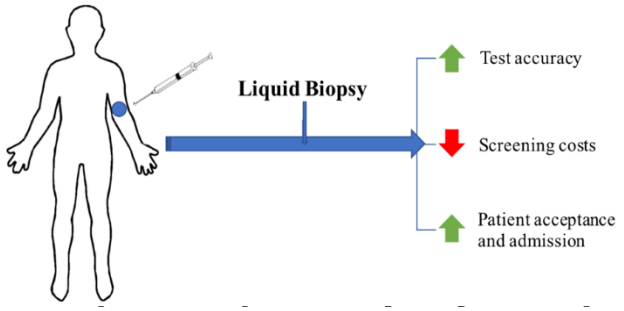
Despite of the economic difficulties in establishing successful cervical screening programs, the women themselves are ultimately responsible for screening and prevention. However, women may be prevented from seeking screening services due to lack of education and various psychosocial or cultural barriers. A comprehensive study surveyed women's attitudes toward cervical screening in rural Kerala, India. The authors found that 89.2 % of participants did not know any risk factors to cervical cancer [35]. Furthermore, 51.4% of participants were unaware of Pap testing and were not motivated to perform testing [35]. In many communities of developing countries, especially rural communities, health education (and education in general) is low among women. This poses a significant limitation for women to seek health diagnostic services. In addition, participants were also prevented from screening due to psychosocial or cultural barriers. In a study of cervical screening in northern Peru, authors found that unscreened women were more likely to believe screening is an evil practice ($P<0.0001$) [36]. Gender inequality is also an key obstacle to screening. Unscreened women expressed more concerns about a male healthcare provider and were more likely to refuse examination [36].

Liquid biopsies for CC must consider all these factors prior to clinical implementation in those countries. But due to its high sensitivity and non-invasive nature, the technology may alleviate some issues of current screening programs. Currently, the sensitivity of non-cervical cancer liquid biopsies ranges from 86% to over 98% when the liquid biopsy and tissue are collected less than six months apart [37]. This particularly benefits women who do just one test in their life. In addition, the use of saliva and urine allows multiple samples to be taken, further improving test accuracy and reducing costs. Women can also perform self-sampling at home, which decreases the need for specialized training and increases acceptance towards screening. Due to these advantages, some developing countries have shown interest in liquid biopsies for screening infectious viral diseases. One case is liquid biopsies for Ebola virus screening in Africa. Balcioglu *et al.* developed a rapid, urine-based liquid biopsy for Ebola early-stage diagnosis. Authors detected four known Ebola biomarkers using a highly sensitive colorimetric assay that is detectable by eye [38]. Since no sophisticated equipment was used, this assay is highly applicable for on-site diagnostics in low-resource nations [38]. Liquid biopsies for CC can be conducted similarly to provide an efficient, low cost alternative to traditional screening approaches.

Traditional CC screening pose significant socio-economic limitations to both health-care systems and patients in developing countries. Liquid biopsies hold considerable promise as an alternative for the clinic due to its various benefits. As well, it can be used for screening of other related cancers, which open new avenues for liquid biopsy research.

SUMMARY AND CONCLUSION

Liquid biopsy is a novel diagnostic and screening tool that has great potential for early detection and monitoring of CC. It is non-invasive, easily repeatable, and highly sensitive for CC biomarker detection. If exosome-based approaches are used, a wide variety of biofluids—from saliva to amniotic fluid to urine—can be used as samples. This can enhance patient admission to regular cervical screening, and help reduce the psychosocial or cultural barriers currently faced by women in developing countries. As such, liquid biopsy is a viable surrogate for traditional Pap and HPV testing. See Figure 4 that integrates the three research questions investigated in this article. Liquid biopsy can potentially screen for other HPV-associated cancers. HPV infection is implicated in 90% of all anal cancers, 70% of all vaginal cancers, and 70% of all oropharyngeal (back of the throat) cancers, affecting both women and men [39]. Like CC, these cancers do not have any



biopsy implementation can improve screening efficacy and acceptance by elevating many of those obstacles.

noticeable symptoms until they develop into advance stage. Therefore, it is important to use liquid biopsies for early prognosis and detection of patients most at-risk of HPV infection.

Despite of the potential of liquid biopsies, many hurdles must be overcome before bringing the technology to the clinic. One of the key concern in this field is the lack of technique standardization. Current techniques to analyze liquid biopsy include BEAMing, NGS, MAP (MIDI-activated pyrophosphorolysis), and mass spectrometry [40]. Each technique detects biomarkers at varying degrees of accuracy, and when combined with different extraction kits, the differences between techniques become even greater. The cost of each technique for the clinic is also an major challenge, especially for low-resource areas. Consider BEAMing as an example. BEAMing requires the use of emulsion PCR, magnetic beads, and flow cytometry, which are all expensive equipment for a clinic in rural India. As well, BEAMing requires screening of the initial tumor sample to generate personalized probes that detect cancer biomarkers [40]. This technique cannot be used if the initial tumor sample is not available, as in the case for clinics where cervical screening is not extensively performed. However, as patients become increasingly aware of the benefits of screening, this limitation might pose a minor issue.

If these challenges are overcome with future research and development, many benefits lie ahead for liquid biopsies. Gerlinger *et al.* illustrated that the primary tumor evolves over time and demonstrates extensive heterogeneity [41]. Liquid biopsies can monitor this evolution by revealing the complete molecular profile and tracking down genetic changes in cancer through cfDNA allele mutations or miRNA changes in exosomes. This can have profound implications for detection of cancer reoccurrence and treatment resistance. In fact, Diehl *et al* found that by monitoring specific cfDNA mutations of *TP53* and *KRAS* in the plasma of patients who undergone colorectal cancer surgery, they can determine cancer reoccurrence at high certainty [42]. Liquid biopsies can also reveal information about dormant disease, which current Pap testing cannot. In the case of HPV-infection, Pap testing cannot identify HPV if the patient fails to develop noticeable symptoms. In contrast, liquid biopsy can potentially identify HPV genes in cfDNA to detect latent HPV infection, thereby allowing clinicians to recommend follow-up and treatment for those patients.

An exciting future extension of liquid biopsy is next-generation biobanking. Traditional biobanks contain only primary tumor samples that are analysed in-house without much multi-disciplinary collaboration [43]. Next-generation biobanks contain liquid biopsy specimens that are collected at different time-points and are analyzed by the collaboration between industry and fundamental researchers [44]. Sample collection at distinct time-points allows for molecular analysis of recurrent or metastatic tumours, which aids in discovery of biomarkers associated with specific treatment regimens. This may lead the way for personalized treatment plans. With partnership between academia and biopharma, the costs for establishing and maintaining biobanks are greatly reduced. One such biobank that showcase the close collaboration across disciplines is the Quebec Clinical Research Organization in Cancer (Q-CROC). Q-CROC partnered up with major pharmaceutical companies, such as Merck, to create multicenter hospital-based infrastructure to house next-generation biospecimens for biomarker discovery [45]. Next-generation biobanking enables wide-use of liquid biopsies for both clinical diagnosis and research. In the future, low-resource nations can access high-quality information from those biobanks to reduce time and costs of screening. With advancements in liquid biopsy, personalized therapies will be accessible to patients across the world.

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