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Exosome-Based Early Detection of Cancer and Parkinson's Disease

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Abstract

Exosome-Based Early Detection of Cancer and Parkinson Disease

by

Haofan Sun

Exosomes have emerged as novel biomarkers for disease diagnostics and prognosis. Exosomes are present in bodily fluids and closely resemble the contents of their parental cells; thus, they have a huge potential to serve as a liquid biopsy tool in the diagnosis of multiple diseases. In particular, tumor exosomes have the potential as biomarkers for the early detection of cancer since their contents reflect the genomic and metabolic abnormalities in their parental cells. With the development of techniques for high-throughput purification and isolation of exosomes and exosome content analysis, exosomal proteins is rapidly becoming an important tool for the early diagnosis of cancer.

Exosomes are extracellular vesicles with a diameter of 30 - 150 nm. These nanovesicles are produced by almost all types of mammalian cells and cancer cells through fusion of an intermediate endocytic compartment, namely, multivesicular bodies, with the plasma membrane.

In the introduction part of this thesis, I introduce the concept of the exosome, its definition, biogenesis and characteristics, and composition. Then I discuss the use of exosomes for cancer prognosis, and α-synuclein circulating exosomes as an example to explain how exosomal proteins work and its high specificity to Parkinson Disease. Finally, I discuss the different methods of isolating and collecting specific types of exosomes.

One unanswered question is at what stage of disease and cancer development can the exosome-based method be useful for diagnosis. To test the practicability of exosome-based methods in cancer and diseases diagnosis, I will establish a mathematical model and analytically calculate the concentrations of cancer-specific exosomes based on tumor growth. Then I will predict how early the exosome-based method can detect cancer and other diseases considering the detection limits of current diagnostic technologies. In addition, I will introduce and discuss the parameters required to inspect and verify the feasibility of my mathematical model. This thesis is focused on two specific exosomes: α-synuclein circulating exosomes for Parkinson disease and HSP70 proteins for liver cancer.

After the exosomes are extracted and purified, they can be resuspended into a small volume solution. Therefore, exosomal samples yield higher concentrations of biomarkers in a resuspension solution than those in blood. In the following, we show that it takes about 2.05 years for a parental cell to expand to a cell population that can secrete a baseline value of the α-synuclein circulating exosomes in Parkinson disease (PD) patients, and 0.73 years for a parental tumor cell to expand to a cell population that can secrete the baseline number of HSP70 proteins circulating exosomes in liver cancer patients. In conclusion, the mathematical model I established can help us predict how exosomal protein can be used to detect cancer and PD.

This thesis is dedicated to my parents, Jiankai Sun and Xiufen Hang, who love me, believe in me, inspire me and have supported me every step of the way.

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Finally, thank you to my friends, family members and people surrounding me in Santa Cruz. Thank you for everything; life is beautiful so enjoy it.

Chapter 1

Introduction and Background

1.1 Motivation

Exosomes have recently gained significant attention as novel biomarkers for disease diagnosis and prognosis. Exosomes, carrying contents of their parental cells, have a huge potential to serve as a liquid biopsy tool in the diagnosis of multiple diseases [2, 3]. Tumor cell-secreted exosomes could be employed as biomarkers for early diagnosis of cancer, because their contents generally reflect the genomic and metabolic abnormalities of the parental cancer cells [4-6]. However, the correlation between the exosome concentration in the blood and the tumor size remains uninvestigated. Several biological parameters related to early tumor biomarker shedding events, such as the biomarker secretion rate, biomarker-shedding rate, number of tumor blood vessel biomarkers and the growth rates of various solid tumors, have not been completed evaluated.

To address this issue, we developed a mathematical model relating the blood exosome levels with disease tissue growth to predict how early one can

detect the disease using exosomal proteins. We collected our model parameters from experimental studies reported in literature.

1.2 Exosomes

1.2.1 Definition of exosomes

Exosomes are small extracellular nanovesicles (30–150 nm diameter) produced by various normal cells and abnormal cells through fusion of an intermediate endocytic compartment, namely, the multivesicular endosome (MVE), with the plasma membrane.

1.2.2 Biogenesis and characteristics of exosomes

Studies have reported that exosomes are produced by various cells during both normal and pathological conditions. Exosomes can be detected *in vivo* and *in vitro* experiments. Exosomes were initially identified in mammalian reticulocytes, which are maturing red blood cells [7]. Subsequent studies have detected exosome secretion from various normal mammalian cells, including dendritic cells, T cells, B cells, mesenchymal stem cells, endothelial cells, epithelial cells and astrocytes, as well as cancer cells. Exosomes are found in most body fluids, including blood, urine and breast milk [7]. In particular, cancer cells can secrete a greater number of exosomes than normal cells [8, 9].

In order to understand the biology of the exosome, we first need to discuss what endosomes are. The endosome is a small intracellular body that is formed when a eukaryotic cell engulfs a small amount of intracellular fluid [9, 10]. The late-stage endosomes are characterized by intraluminal vesicle (ILV) formation. Briefly, endosomal membranes bud inward to form invaginating membranes that randomly encapsulate cytoplasmic contents, transmembrane proteins and peripheral proteins. ILVs have varying diameters in between 30–100 nm [11]. Late stage endosomes undergo morphological changes. Early endosomes are tube-like structures that usually form close to the cytoplasmic side of the cell membrane, whereas late endosomes are hollow-sphere structures located closer to the nucleus. Late endosomes are also known as MVEs [7].

In general, MVEs may fuse with lysosomes and degrade their contents through hydrolysis inside the lysosome. Alternatively, MVEs may also release ILVs into extracellular environments through exocytosis. Therefore, these vesicles are named “exosomes”.

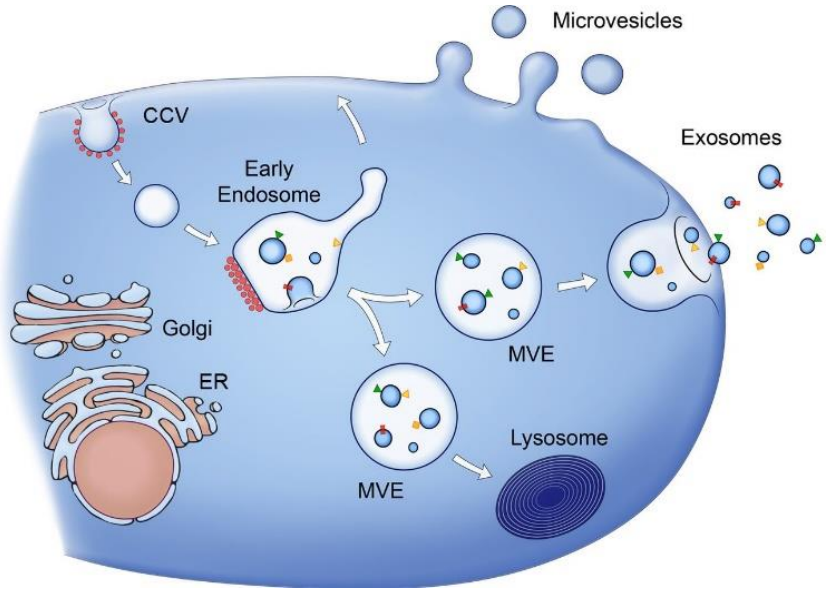

Figure 1–1. Formation of MVEs and exosomes. Plasma membranes bud inward to form microvesicles. Exosomes are small vesicles that are released through exocytosis of MVEs. Transmembrane proteins and peripheral proteins are shown. (Adapted from [11])

Exosomes are a major type of extracellular vehicle (EV). Processes that are involved in the secretion of exosomes are illustrated in figure 1-1. Exosome release can be summarized into three steps: formation of intraluminal vesicles (ILVs) in MVEs, transport of MVEs to the plasma membrane and exocytosis of MVEs.

1.2.3 Composition of exosomes

Exosomal content is abundant and varies significantly. With continuous updates from scientists and researchers, Exocarta and Vesiclepedia provides a comprehensive database for researchers [12]. Exosomes are composed of

proteins, DNAs, RNAs (mRNAs, miRNAs, tRNAs and rRNAs) and lipids (Figure 1-2) [13]. Both databases include protein, nucleic acid and lipid data.

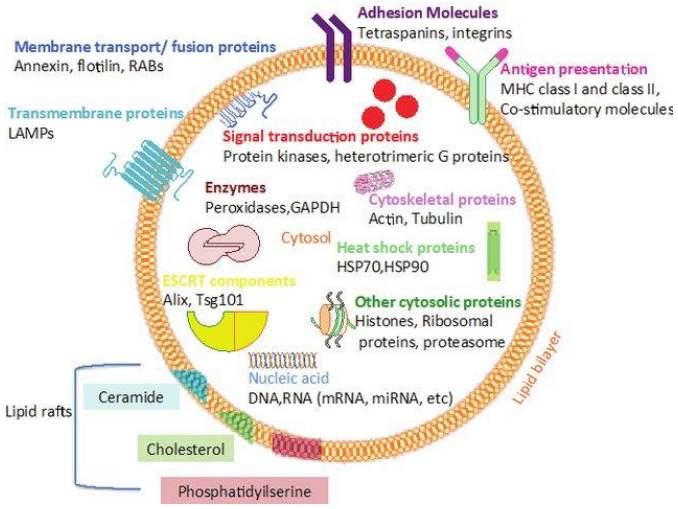

Figure 1-2. Composition of cell-derived exosomes. Exosomes contain proteins, DNAs, RNAs and lipids reflecting their parental cells [12].

Proteins. Almost all exosomes contain proteins of the heat shock protein family (HSPs) such as HSP 70 and HSP 90 [14, 15]. In addition, exosomes are rich in proteins of the tetraspanin family such as CD9, CD63, CD81 and CD8. Exosomal proteins also present some unique characteristics compared to traditional serum biomarkers. First, some exosomal proteins show higher specificity for diseases compared to secretory proteins. For example, glypican-1 (GPC1) is particularly abundant in cancer-derived exosomes, showing better specificity than CA-199 or serum-free GPC1 (100% vs 79.49% vs 82.14%) in

distinguishing non-cancer subjects from pancreas cancer patients [5]. In addition, HSP70 is 3–4 times more abundant in exosomes derived from liver cancer cells than in those derived from non-cancer cells [9]. Second, exosomal proteins are highly stable; the bilayered exosome membrane protects exosomal proteins from external proteases and other enzymes from degradation in the bloodstream [16]. To date more than 41,860 different exosomal proteins have been reported and listed in online databases.

Nucleic acids. Exosomes carry different types of RNAs, such as messenger RNA (mRNA), small non-coding RNA (miRNA), and long non-coding RNA (lncRNA). Exosomes deliver intact mRNAs to recipient cells to directly modulate the mRNA level of a certain gene. Therefore, exosome-mediated mRNA transfer represents a form of crosstalk between cells. The miRNAs are 21–25 nucleotide-long small non-coding RNAs that regulate gene expression at the posttranscriptional level by base-pairing with target mRNAs. The miRNA–mRNA interactions result in a reduced translation efficiency or a decreased stability of target mRNAs [16].

Lipids. Lipids constitute a big and varied family of naturally occurring organic compounds that are soluble in nonpolar organic solvents; in general, lipids are

insoluble in water. Different lipids demonstrate variable structural stability. Several studies have reported that certain types of lipids are highly enriched in exosomal membranes [17]. One study quantified approximately 280 lipid species belonging to 18 classes in exosomes isolated from a prostatic carcinoma cell line (PC-3) [18]. Another study identified more than 500 lipid species in exosomes isolated from a human colon carcinoma cell line (LIM1215) [18]. According to results reported by Haraszti *et al.*, there were great differences in the lipid components of exosomes and macrovesicles derived from U87 glioblastoma cells, the human liver cell line Huh7, mesenchymal stem cells (MSCs) from bone marrow [17].

1.3 Exosomes in cancer

Exosomes, once regarded as artifacts or cellular trash, have recently emerged as novel biomarkers, especially for cancer diagnosis, with an emphasis on investigating the physiological mechanism of specific exosomal components, including proteins, RNAs and DNAs.

1.3.1 Specific exosomal proteins for cancer detection

Exosomes contain specific cellular proteins on their membrane surfaces that can be used as biomarkers for cancer diagnosis and prognosis [19]. They present some unique advantages for clinical diagnostics. Their small sizes allow them to penetrate physiological barriers: exosomes are present almost all types of bodily fluids [20].

Equally important, blood is a complex biological mixture of proteins, vesicles and cells. Proteins secreted from abnormal cells have a low concentration after collected from 3-liters of blood. Hence, biomarker proteins cannot be readily detected during the early stages of a disease, when the secretion rates of biomarkers are low. However, in normal cells, there are more than 10^9 exosomes in each milliliter of human blood, and abnormal cells (i.e., tumor cells) can secrete at least 10-fold more exosomes. Thus, we can obtain an impressive number of exosomes from cancer cells after purification. Accodingly, exosomes present huge potential as efficient biomarkers that can be used to diagnose early stages diseases including cancer and PD [21].

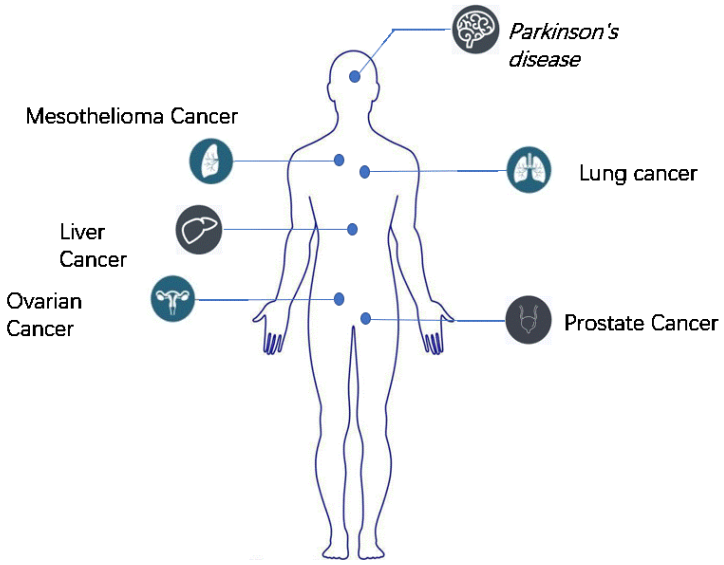

Figure 1-3. Specific exosomes used for diagnostics of PD and different cancer diseases.

Figure 1-3 shows some of the current diseases that have been targeted for exosome-based early diagnosis. A number of studies have reported that specific exosomal proteins can be used as biomarkers for different cancer types. For example, latent membrane protein 1 (LMP1) was detected in exosomes isolated from Epstein Barr virus (EBV)-infected nasopharyngeal carcinoma (NPC) cells [22]. Furthermore, exosomal glypican-1 (GPC1) was used as a biomarker in the early diagnosis of pancreatic cancer [5]. In a proteomic profiling study involving [23], prostate cancer gene-3 (PCA-3), β-catenin and other prostate cancer biomarkers in exosomes isolated from the urine of prostate cancer patients. In the early diagnosis of ovary cancer,

epithelial cell adhesion molecule (EpCAM) and CD24 were demonstrated to be promising exosomal biomarkers [22, 26]. Various highly concentrated exosomal proteins for different cancer types are listed in Table 1-1.

Table 1-1. Exosomal proteins used for cancer and PD diagnosis

Cancer and disease	Exosomal Proteins	Reference
Pancreatic	**Glypican-1**	**[5, 24]**
Prostate	**PSA**	**[23]**
Ovarian	**CD24, EpCAM, CA-125**	**[25]**
HCC (Liver cancer)	**HSP70**	**[26]**
Parkinson's disease	**α-Synuclein**	**[27]**
Lung	**NY-ESO-1**	**[28]**
Breast	**PKG1, RALGAPA2, NFX1, TJP2**	**[29]**

1.3.2 α-Synuclein exosomal proteins for Parkinson's disease detection

Given its importance in the development of Parkinson's disease, recent studies have shown that α-synuclein in cerebrospinal fluid (CSF) can be used as a biomarker for diagnosing and monitoring PD [30]. However, soluble α-synuclein is present in the blood, plasma and serum at very low concentrations. In contrast, a case-control study on 267 PD patients and 215 healthy controls

suggested that the α-synuclein level is significantly higher in exosomes isolated from plasma of PD patients than in those isolated from healthy controls [31]. Furthermore, the sensitivity and specificity of the plasma exosomal α-synuclein biomarker are similar to those of the CSF α-synuclein biomarker in diagnosing and monitoring PD. In this thesis, we focus on estimating the CSF exosomal α-synuclein levels, since the mechanism underlying the transport of α-synuclein-containing exosomes to plasma remains uninvestigated [31].

1.4 Isolation and collection of exosomes

Typically, the enrichment of exosomes involves isolation from the blood or CSF (in the case of PD), and re-suspension in a small volume of solution.

1.4.1 Ultracentrifugation-based isolation techniques

The most commonly used method for isolation is ultracentrifugation, which uses extremely high centrifugal acceleration that reach 100,000 × g. It is extensively used for the separation and purification of particulate materials, including microbial cells, cellular organelles and vesicles. Because of the strong centrifugal forces, small particles can be sedimented into different layers according to their size, density, and shape in suspension. Ultracentrifugation-based exosome isolation is the most effective and commonly used exosome

isolation method. Ultracentrifugation-based exosome isolation has been used in nearly 56% of exosome-related studies [32].

1.4.2 Other methods to isolate exosomes

In addition to ultracentrifugation, filtration, molecular sieve chromatography, affinity chromatography, polymeric precipitation and microfluidics are also documented exosome isolation methods [32-35]. However, impurities, such as cell culture media, biological fluids and other lipid structures can be introduced when these methods are used.

1.4.3 Isolation process of exosomes

Ultracentrifugation-based exosome isolation always includes several rounds of centrifugation using different centrifugal forces (~100,000–120,000 × g) and for different durations. After centrifugation, solutes of different sedimentation coefficients are separated into different layers in the gradient medium. The density of exosomes ranges from 1.10 to 1.21 g/ml. Therefore, the centrifugation medium aliquots containing exosomes can be easily obtained by selective collection from the corresponding density regions. The aliquots are then briefly ultracentrifuged at ~100,000 × g, and the exosome pellet is re-suspended in a saline buffer [33].

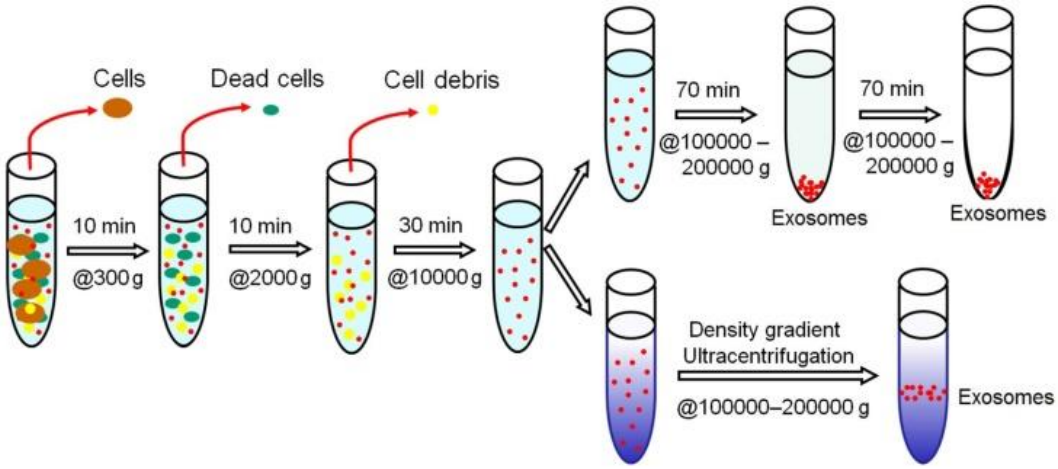

Figure 1-4: Process of differential ultracentrifugation-based exosome isolation. At the beginning, a cleaning step is used to remove large bioparticles such as cells. Subsequently, protease inhibitors are added to avoid exosomal protein degradation during the isolation. Exosomes may be in supernatant fractions or pellet fractions depending on the centrifugal force. For further purification, exosomes are re-suspended in an appropriate solution (such as PBS) and centrifuged at a higher speed. Finally, the isolated and purifed exosomes are stored at -80°C for subsequent studies. (Adapted from [33])

1.5 Summary

Traditional blood biomarkers are diluted in human blood, whereas exosomes can be isolated and enriched from various body fluids. After re-suspending exosomes in a small volume of solution, it is possible to obtain exosomal proteins in high concentrations for analysis. It is predicted that the concentration of biomarkers can be enriched more than three orders of magnitude using exosomes isolation and enrichment techniques.

Chapter 2

Methods

The mathematical model and tumor growth kinetics

To estimate the time required for early detection of cancer and Parkinson's disease, which is to quantify the amount of time needed to growing cancer cell and diseases cell line population to secrete detectable amount of exosomes and exosomal protein, we developed a mathematical model based on an earlier study on ovarian cancer detection with CA125 shedding [34]. We obtained the tumor growth and exosome shedding rates from published literature and utilized our model to predict the early detection of cancer using exosomal proteins. Our model shows which parameters have the largest impact on early cancer diagnosis. In addition, we also show how changing each baseline parameter value affect our ability detect dieases early.

2.1 Cancer cell growth model

2.1.1 Gompertzian model equation

Biomarkers are usually evenly distributed in body fluids. Therefore, one-compartment models can be used to describe biomarker distributions (Fig. 2-

1). In this study, we only focus on the plasma biomarkers derived from abnormal cells and neglect the biomarkers originated from the health cells .

The plasma biomarker influx represents the total level of biomarkers derived from abnormal cells, $\mathrm{U}_T(t)$, or from normal cells, $\mathrm{U}_H(t)$. Both values are functions of the following argument: (a) $\mathrm{f}_{PL,T}$ represents the fraction of the biomarkers entering to tumor vessels. $\mathrm{f}_{PL,H}$ represents the fraction of the biomarkers entering the vessels of normal tissues. These two values may be different since tumor vessels are more permeable, and the biomarkers may be degraded within the tumor microenvironment. (b) R_T represents the total amount of the biomarkers released by an abnormal cells per unit time. R_H represents the total amount of the biomarkers released by a normal cell per unit time. In this study, we assumed that both R_T and R_H were constants. (c) $\mathrm{N}_T(t)$ represents the total number of abnormal cells that can release biomarkers at time t. If a biomarker is only released by some of the abnormal cells, $\mathrm{N}_T(t)$ may only account for a fraction of the abnormal cells. N_H represents the total number of normal cells that are releasing biomarkers. We assume that the number of normal cells does not change with time.

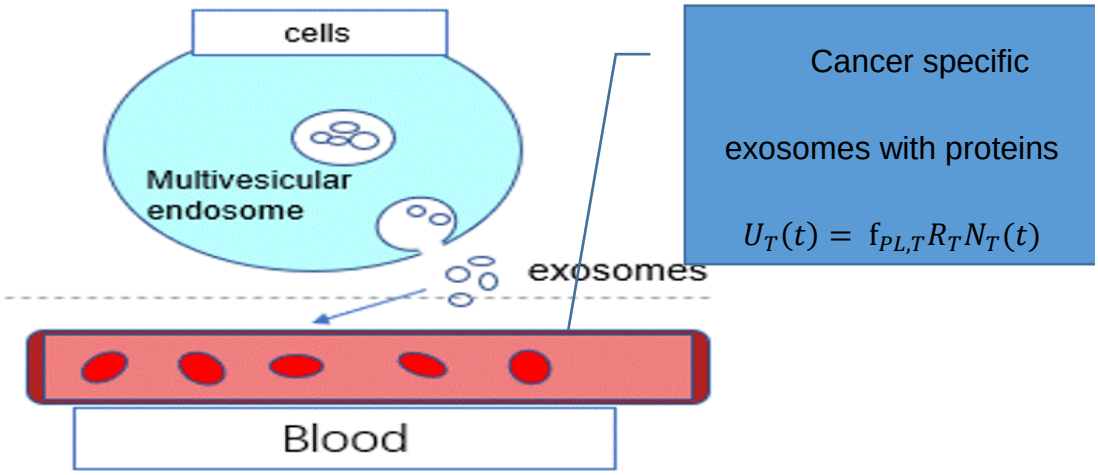

Figure 2-1. Schematic diagram of tumor cells releasing exosomes. q_{PL} represents the number of biomarker-containing exosomes released by abnormal cells into serum or plasma. All the parameters are presented in Table 1.

Time evolution of biomarker-containing exosome numbers can be described using the following equation:

$$\frac{dq_{PL}(t)}{dt} = u_T(t) + u_H(t) - k_{EL} q_{PL}(t) \qquad (2\text{-}1)$$

In the above equation,$u_T(t)$represents the number of biomarker-containing exosomes shed by abnormal cells, $u_H(t)$ represents the number of biomarker-containing exosomes shed by normal cells and $k_{EL} q_{PL}(t)$ represents the number of biomarker-containing exosomes discharged from the plasma.

The rate at which abnormal cells derived exosomes are released into the serum is

$$u_T(t) = f_{PL,T} R_T N_T(t) \tag{2-2}$$

Similarly, the rate of exosome release from healthy cells is

$$u_H(t) = f_{PL,H} R_H N_{H,0} \tag{2-3}$$

The growth of abnormal cells can be simulated by the Gompertz equation,

$$N_T(t) = N_{T,0} e^{\frac{k_{GR}}{k_{decay}}\left(1-e^{-k_{decay}t}\right)} \tag{2-4}$$

Combining the equations above, one can show that

$$\frac{dq_{PL}(t)}{dt} = f_{PL,T} R_T N_{T,0} e^{\frac{k_{GR}}{k_{decay}}\left(1-e^{-k_{decay}t}\right)} + f_{PL,H} R_H N_{H,0} - k_{EL} q_{PL}(t) \tag{2-5}$$

This equation is solved numerically using MATLAB.

2.1.2 Simplified the G model

In an earlier study using clinical samples (267 PD, 215 controls), it was found that the exosomal α-synuclein level was 3–4 times higher in the plasma of PD patients than those of the healthy controls, suggesting increased α-synuclein efflux into the peripheral blood in PD patients [31].

Therefore, in our mathematical model on the α-synuclein exosomes, the amount of exosomes released by normal cells was neglected.

Equations 2–5 can then be simplified as

$$\frac{dq_{PL}(t)}{dt} = f_{PL,T} R_T N_{T,0} e^{\frac{k_{GR}}{k_{decay}}\left(1-e^{-k_{decay}t}\right)} - k_{EL} q_{PL}(t) \tag{2-6}$$

2.2 Concentration of re-suspension solution and final exosomes

We can then calculate the concentration of the biomarkers in the blood (C) based on the amount of the abnormal cell derived biomarker-containing exosomes (q_{PL}) in serum.

$$C = \frac{q_{PL} \times N_G}{V} \tag{2-7}$$

V is the total volume of serum (approximately 3.15 L) [10].

N_G is the number of biomarkers per exosomes [9], [14-16].

We can then calculate the concentration of α-synuclein and HSP70 proteins in the re-suspension (C_r).

$$C_r = \frac{C \times r \times V_t}{V_s} \tag{2-8}$$

r is the exosome recovery rate. Since a small proportion of exosomes will be damaged during isolation, only a certain proportion of exosomes can be successfully isolated. In this study, we assume r is 70% [17].

V_t is the blood sample volume. Usually 10 ml of blood is used for blood tests.

V_s is the volume of re-suspension, ranges from 10 μL to 10 ml based on current technology.

With the calculated biomarker concentration C_r and the actual biomarker concentration d_c, we can calculate the final detection time.

Table 1. Description of parameters involved in the abnormal cell-derived exosome model. Parkinson's disease α-synuclein circulating exosomes

Parameter	Description (units)	Baseline value	Range simulated	Reference
$f_{PL,T}$	Fraction of exosomes entering CSF	0.1	0.01 to 1	[34, 35]
R_T	Exosome secretion rate per abnormal cell (# /day/cell)	45	10^{-3} to 10^{3}	[27],[31]
$N_{T,0}$	Initial number of abnormal cells	1	1 to 10^{10}	—
k_{GR}	Growth rate to abnormal cell population (/day)	0.015	0.01 to 0.1	[27, 36]
k_{EL}	Elimination rate of exosome from plasma (/day)	0.46	0.01 to 21.8	[25], [37]
k_{decay}	Rate at which abnormal growth rate decrease (/day)	1×10^{-4}	10^{-6} to 10^{3}	—
d_E	Detection limit for number of exosomes (/ml)	1×10^{5}	10^{3} to 10^{12}	—
N_G	Number of biomarkers per exosomes	450	10^{1} to 10^{2}	[9, 27, 35]

Table 2. Description of parameters involved in the cancer-derived exosome model. HCC (Liver cancer) HSP70 proteins circulating exosomes

Parameter	Description (units)	Baseline value	Range simulated	Reference
$f_{PL,T}$	Fraction of exosomes entering serum	0.1	0.01 to 1	[35],[9]
R_T	Exosomes secretion rate per tumor cell (# /day/cell)	475	10^{-3} to 10^{3}	[26]
$N_{T,0}$	Initial number of tumor cells	1	1 to 10^{10}	—
k_{GR}	Growth rate to tumor cell population (/day)	0.036	0.01 to 0.1	[38]
k_{EL}	Elimination rate of exosome from serum (/day)	0.29	0.01 to 21.8	[38]
k_{decay}	Rate at which tumor growth rate decrease (/day)	1×10^{-4}	10^{-6} to 10^{3}	—
d_E	Detection limit for number of exosomes (/ml)	1×10^{5}	10^{3} to 10^{12}	—
N_G	Number of biomarkers per exosomes	195	10^{1} to 10^{2}	[26, 39-42]

2.3 Data description

$f_{PL,T}$ is the proportion of exosomes released into serum, Plasma or CSF

Based on the reference discretion for HCC cells (liver cancer cell line), only 10% of cancer-derived exosomes are released into serum. The remaining 90% undergo endocytosis and mediate intercellular communication [22, 23]. As for Parkinson's disease, the estimated fraction of exosomes entering plasma is also ~10% [27].

R_T is the number of exosomes released by an abnormal cell per unit time (secretion rate).

In accordance with the NTA analysis of particle diameters in exosomes isolated from SH-SY5Y cells, the number of α-synuclein circulating particles was 4.5×10^6/ml, which was obtained from the α-synuclein over-expressing SH-SY5Y cells in 24 h, since the concentration of the cancer cells was 1×10^5/ml, a total of 45 exosomes were released by each SH-SY5Y cell per day.

According to the ExoELISA kit calculation, the number of HepG2-exosomes in the cultured media was 2.37×10^8/ml (the HepG2 cells ($5\times10^5/\mathrm{ml}$) had been incubated for 96 h) [26]. Thus, the secretion rate of the HepG2-exosomes was 475.

$N_{T,0}$ is the initial abnormal cells number

We assume that all the abnormal cells originated from one parental cell. The value of $N_{T,0}$ represents the number of abnormal cells at the beginning.

k_{GR} is the abnormal cells growth rate

The initial number of H-SY5Y cells was 1-1.5×10^6/ml. The cells were seeded at 2×10^4/ml and then incubated for 48 h [3]. Thus, we determined that the growth rate for α-synuclein-expressing SH-SY5Y cells was 0.02–0.013 per day.

The initial number of HepG2 cells was 6×10^6/ml. The HepG2 cell growth rate was 1,815/h [38]. Thus, we determined that the growth rate for HepG2 cells is 0.036 per day.

k_{EL} is the amount of biomarkers eliminated from serum per day (elimination rate).

Each protein has its specific half-life in serum [25]. If a protein loses its activity, it can no longer be used as a biomarker. The elimination rate was ~0.46 for Parkinson's disease and ~0.29 for liver cancer exosomes. According to our estimates, the maximum elimination rate was 21.8, which is approximately the endocytosis rate of exosomes.

d_E is the detection limit of the exosome concentration

Current technology for protein's concentration detection limit ranges from 100 aM to 100 pM, as shown in figure 2-2. In order to get the final protein concentrations within this range, we followed an inverse process to calculate corresponding exosomal concentration requirement. We found that the detection limit of the concentration of exosomes was between 10^2 to 10^7 number/ml.

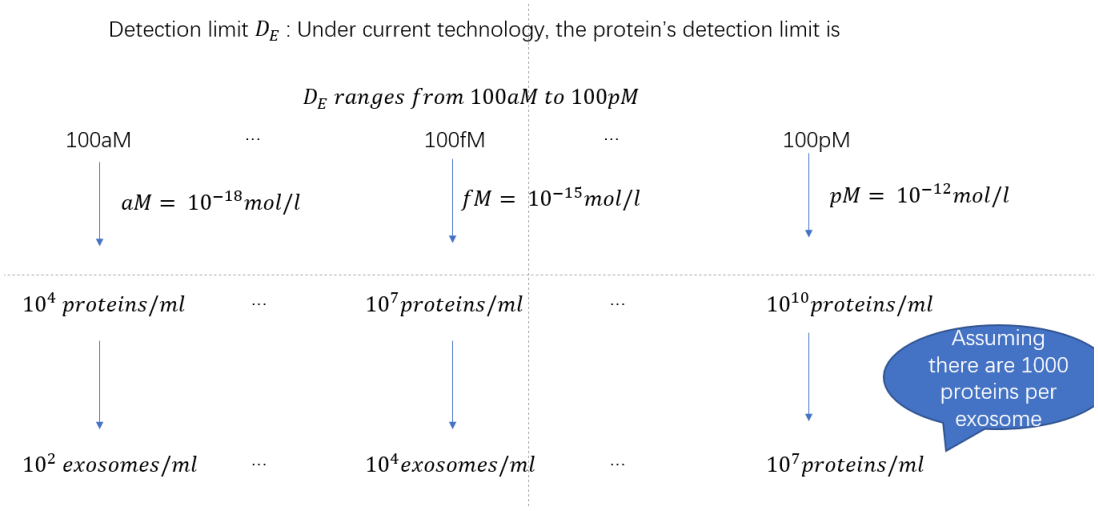

Figure 2-2. Derivation of valid exosomes concentration boundary

N_G is the number of biomarkers per exosome

The α-synuclein concentration is 10–25 pg/ml [2-3], and the protein molecular weight for α-synuclein is 16 kDa [1-2]. If we set the α-synuclein concentration to be 12 pg/ml, we estimate that there are 450 molecules of α-synuclein per exosome.

In HepG2 cells, ~25% of the exosomes expressed HSP70 [26]. The protein molecular weights of CD63, CD81, NKG2D and HSP70 are 30–65 kDa, 20–25 kDa, 27–40 kDa, and 50–75 kDa, respectively [41]. The protein concentrations of the HepG2 – exosomes were 664.2 μg/ml; thus, by using other parameter base values, we calculated the Hsp70 concentration as 90 pg/ml and estimated that there were 195 molecules of Hsp70 per exosome.

Chapter 3

Results and Discussion

3.1 Estimate detection time using G model

Figure 3-1 displays the estimated detection time curve for the two diseases. For exosome concentrations to reach detectable levels, which is 10^5 number of particles/ml, 750 days (around 2.05 years) is needed for the Parkinson's disease and 283 days (0.73 year) is needed to for the liver cancer.

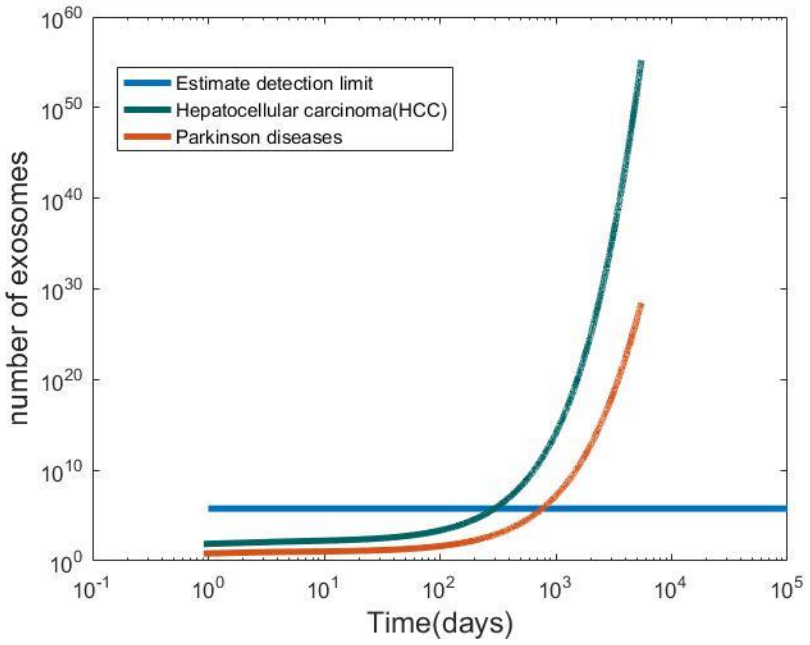

Figure 3-1: Cumulative curve of cancer-derived exosomes. The orange line (——) represents the number of tumor-derived α-synuclein-containing exosomes in plasma. The green line (——) represents the number of tumor-derived HSP70-containing exosomes in plasma. The blue line (——) represents the estimated detection limit for determining exosome numbers.

3.2 Different parameters influence the detection limit

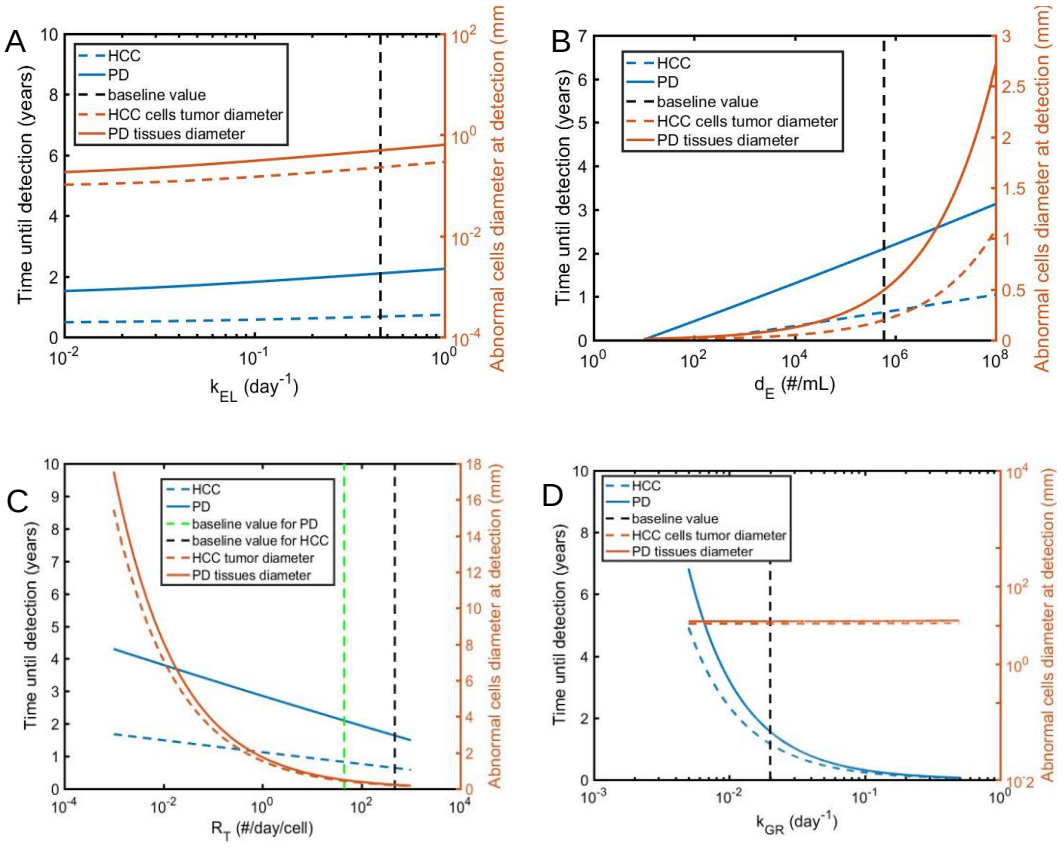

Figure 3-2. The shortest tumor growth duration and minimum tumor size required for exosomal biomarker detection. The earlist detection time **(**T_D, left y-axis) is shown for HCC (---) and Parkinson's disease (——). The minimum abnormal tissue size for exosomal biomarker detection (right y-axis; HCC (---), Parkinson's disease (——) was estimated based on the assumption that one cubic millimeter of abnormal tissue contains 10^6 abnormal cells. Baseline parameters were indicated by the black dashed vertical lines. Detection time and tumor size is calculated for varying (A) k_{EL} the elimination rate of biomarker from serum. (B) d_E detection limit for determining exosome numbers, (C) R_T the number of exosomes released by a tumor cell per unit time (secretion rate) and (D) k_{GR} the growth rate of the tumor cell population.

Our sensitivity analysis suggests that the cancer cell-related parameters, including R_T, k_{GR}, k_{EL}, d_E, can greatly affect the shortest tumor growth duration. Figure 3-2A shows that k_{EL}, represents the elimination rate of the biomarker from CSF, greatly impacted the estimated PD detection time and abnormal brain tissue diameter. When the base line value for k_{EL} was 0.46, the estimated PD detection time was ~2 years, and the tumor cell diameter was 0.6 mm. For HCC (liver cancer), k_{EL} is the elimination rate of biomarker from serum. When k_{EL} is increased, it has minimal affect on detection time [43].

Figure 3-2B shows that the technological advancement can significantly improve our ability to detect Parkinson's disease. However, we observed that d_E has a small effect on our ability to detect liver cancer early. The detection time only changed slightly with smaller d_E.

As we can see in Figure 3-2C, for liver cancer, when R_T is increased, the estimated detection time of liver cancer decreased significantly, and reached 0.73 years at the baseline value for exosomes secretion rates at 475 particles/day. In that time, the corresponding tumor diameter was ~0.06 mm. As for PD, the estimated detection time of the disease decreased even faster. It took 2.05 years for exosome levels to reach the baseline value assuming that

the exosome secretion rate was 45 number of exosomes particles/day. As for the tumor diameter, it was 0.17 mm at the base line value.

For Figure 3-2D, the growth rate of the tumor cell population k_{GR} had a minor effect on the tumor diameter. As we observe, the changing k_{GR} did not affect the tumor diameter at the detection time. However, it had a huge impact on the cancer detection time, when k_{GR} increased 0.1 per day. The cancer detection time decreased exponentially since in early stage of cancer tumor cells growth is faster. When the tumor growth rate was higher, the number of secreted exosomes were higher, leading to a rapid decrease in the detection time (early detection).

3.3 Re-suspension in vitro

We set the detection limit d_E to be the lowest biomarker concentration that can be detected by ELISA (0.1 pM). Figure 3-3 shows our analysis for *in vitro* detection of exosomal proteins. We assume a plasma solution of 10 ml with 10^5 tumor-derived exosomes each of which carrying 450 α-synuclein proteins. Following the equation (2-7), the concentration of exosomal α-synuclein proteins in this sample is $4.95 \times 10^{-16}\ \mathrm{mol/L}\ (100\ \mathrm{aM})$. If the exosomes are re-suspended in 1 µl of solution, the concentration of α-synuclein proteins will

reach 4.95×10^{-13} mol/L ≈ 0.5 pm (larger than detection limit 0.1 pM), and the detection time is ~2.05 years.

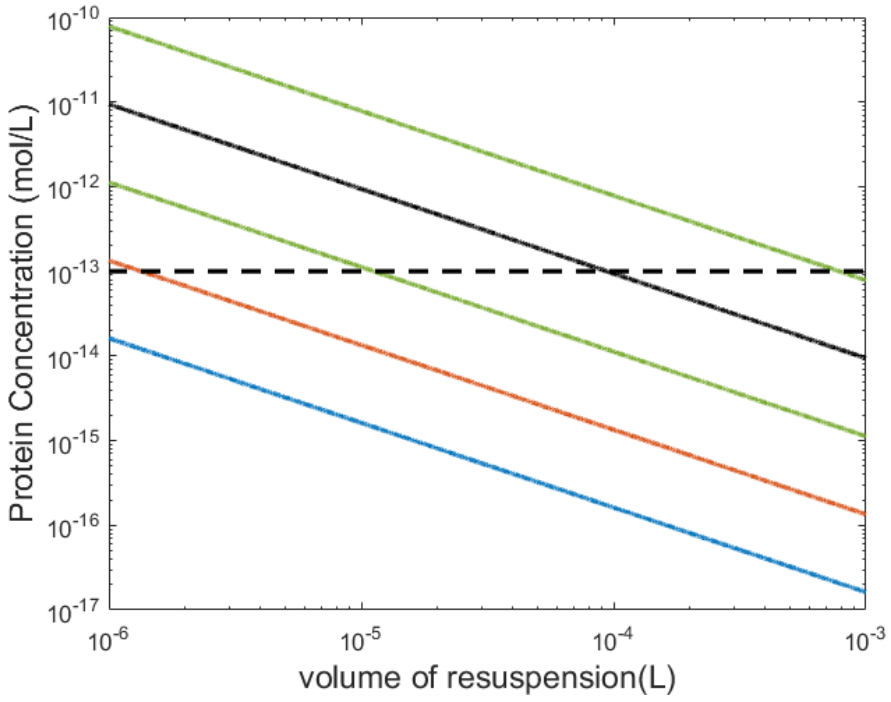

Figure 3-3: Different volumes of solvent with the corresponding detection time. Colored lines indicate different orders of magnitude in the number of tumor-derived serum exosomes. (——) 10^7; (——) 10^6; (——) 10^5; (——) 10^4; (——) 10^3. The dashed vertical line (– – –) represents the baseline value for the protein detection limit.

In order to achieve earlier detection, there are two possible ways one can follow. First, we can use less solution while resuspending isolated exosomes. Second, we can use a method that detects a lower concentration of the biomarkers. In summary, either a lower volume of resuspension V_s or a method with a lower detection limit d_E should be used.

Chapter 4

Discussion and Perspectives

4.1 Capability of decrease detection time

Traditional molecular biomarkers are diluted in V_{PL}= 3.15 L of human blood [43]. Therefore, if we want to detect abnormal cell biomarkers during the earlier stages of a disease, we must enrich the biomarkers. Use of exosomes provides this capability. Exosomes are carriers of a variety of biomarkers. By re-suspending plasma exosomes in a small volume solution, the concentration of target biomarkers can be greatly increased. Therefore, for a given *C* value (blood biomarker concentration), we can detect disease-specific biomarkers at earlier stages by decreasing the volume of the exosome re-suspension (V_s), or increasing the exosome recovery rates (*r*) by optimizing the isolation method. At this point, it is difficult to increase sample volume (V_t) since patients may be reluctant to provide more blood. Therefore, in this study, V_t was constant (10 ml). Additionally, the volume of the exosome re-suspension could not be reduced indefinitely. Furthermore, the biological activities of exosomes and biomarkers should be maintained throughout the process. If we can use ultracentrifugation to obtain exosomes in a small volume (< 1 μl), we can

decrease the detection time in 1–4 or more orders of magnitude. Improving assay detection limit, d_E; however, requires more advanced and accurate technologies.

We also found that detection limits of exosomal biomarkers are impacted by a number of factors. We show that increased abnormal cell biomarker-shedding rate, R_T, or decrease the biomarker elimination rate from plasma, k_{EL}, and increase the fraction of biomarker entering plasma, $f_{PL,T}$ helps our early detection capabilities.

4.2 Perspectives

Using experimental measurements collected from different disease-specific exosomes, our mathematical model is useful in understanding abnormal tissue growth and estimating biomarker concentrations associated to it. In this thesis, we analyzed Parkinson's disease and liver cancer. Further studies are needed to test the validity of this mathematical model with data derived from other studies. We also need to identify additional cancer-specific biomarkers in exosomes. The related data may further prove our mathematical model to study different types of solid tumors and their corresponding diagnostic biomarkers.

In addition, we only focused on cancer-specific exosomal protein biomarkers, but exosomes also carry RNAs and mRNAs, which could be used as molecular biomarkers. Therefore, further studies are needed to understand the potential value of exosomal RNA or mRNA biomarkers.

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