Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Lecture 42 : Liquid biopsies in cancer detection

Namaskar. Welcome back to the NPTEL lecture series comprehensive molecular diagnostics and advanced gene expression analysis. In the last class we have started molecular diagnostics in cancer management, where we have discussed the different molecular genetics or genetic phenomena which are occurring in tumorogenesis. In today's class we are going to discuss one very pertinent topic that is liquid biopsy in cancer detection. So, what are the basic gross concepts which we are going to cover that is the concept of liquid biopsy what liquid biopsy actually is then circulating tumor cells, circulating tumor cells enrichment technologies, circulating tumor DNAs and tumor educated platelets. These are the very important things which are taken care of in liquid biopsy.

So, what basically liquid biopsy is it is the biopsy which is done over the liquid sample. So, what are the liquid sample in our body our body fluids it start it starts from blood CSF urine different tissue fluids. So, liquid biopsy deals with these samples. So, basically in a tumor cell when we collect body fluids and detect some biomarkers which which indicates the presence of cancer or tumor cells that is what is done in liquid biopsy.

So, there are different definitions for liquid biopsy somebody said group said a test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in blood. A test to search for cancer cells or pieces of DNA from tumor cells in blood sample liquid biopsy can serve a variety of purposes. So, these are the different definitions for liquid biopsy, but what is the actual purpose for doing it. So, basically it helps in early detection of cancer, but again it needs high blood volume. Using liquid biopsy tumor staging and monitoring can be done distinguish the differentiation from the patients between low and high risk of recurrence can be done.

Metastatic tumors metastatic progression of the tumor whether there is a chance or not that can be predicted also the treatment efficacy can be monitored via liquid biopsy. So,

what are the biomarkers exactly which are which we see in liquid biopsy sample. So, the very common parameters are circulating tumor cells or CTCs circulating tumor nucleic acids ctNA and also exosomes. Now they are collected from the body fluids like blood, serum, plasma, urine, CSS, saliva. These are the different parameters which we check in liquid biopsy sample coming to one by one.

Now, circulating tumor cells basically these are the cells which represents a very rare population in blood and in present in very low quantity. Similarly it is less than 10 cells per ml in comparison to 1 million WBCs in per ml in our circulation. However, these CTC detection in a routine blood specimen can help in monitoring or in diagnosis of tumor. So, how can we detect this circulating tumor cells definitely via few markers. So, what is exactly a perfect CTC marker? A marker which is only expressed in CTC and not on any other blood cells like leukocytes, endothelial cells, hematopoietic stem cells or mesenchymal stem cells and are when expressed over the CTC they are never repressed during their invasion or the metastasis process.

So, it is one persistent exclusive marker which is present only over circulating tumor cells, but definitely such perfect gold standard markers are not available why I will discuss. So, to assess or to detect the circulating tumor cell one very common technology is adopted that is enrichment process enrich enriching the circulating tumor cells to increase the concentration of CTCs because they are present in a very low count for and thus this enrichment process basically help in detection via different single immunostaining processes. Now how those enrichment processes are done via exploiting certain biological or physical properties of this tumor cells. Now what are the biological properties? Definitely there are few markers protein markers which are present over this circulating tumor cells. So, there are cell surface proteins and against those there are antibody based enrichment methods which where the antibodies are raised against those specific cell surface proteins.

Those antibodies are attached in some columns or micro post or magnetic devices and they capture the circulating tumor cells via antigen antibody reaction. Apart from that there are other subcellular markers which are also used to further characterize the tumor cells. To avoid false positive finding what is done there is confirmatory test via some genomic analysis that is artificial fish, then single cell analysis or by immunostaining of the cancer cells. Similarly, false negative finding can be avoided by using antibody in combination with various other markers which gives the CTCs heterogenicity. So, these are the list of enrichment techniques.

Lets discuss one by one. So, based on the biological properties there can be positive enrichment or negative enrichment. Now what is positive enrichment? Exploiting the tumor exploiting the cell surface markers of tumor cells there is enrichment to increase

the tumor cells count the concentration or density of the tumor cells. So, what are those proteins? They are basically epithelial proteins sometimes they are mesenchymal proteins, sometimes the antibodies are basically targeted both towards it both against and epithelial as well as mesenchymal proteins. Now, what is the negative enrichment? So, what are the negative things which are present in the circulation apart from the tumor cells? Of course, the normal leukocytes.

So, if we somehow deplete the leukocytes normally the tumor cells will be increased in concentration. So, we can deplete the leukocytes via giving via raising antibody against CD45 which is a leukocyte marker. So, the very common epithelial marker which is expressed on circulating tumor cell is epithelial cell adhesion molecule or EpCAM. Also certain cytoskeletal proteins, cytokeratins their variety are also used like cytokeratin 8, 18, 19. Additionally tumor cells which has gone through epithelial to mesenchymal transition remember they expresses the mesenchymal markers also.

So, antibody can be raised again those mesenchymal markers like cadherins, n-cadherins and y-mentins. So, these are the markers or antibodies based on which protein and antigen antibody interaction based positive enrichment can be done. Now apart from the biological properties even the physical properties for circulating tumor cells can be different from other cells in circulation like leukocytes or hematopoietic cells or endothelial cells. So, for separation or for enrichment those physical properties can also be exploited like filtration method where a membrane and filtration based system is adopted to discriminate the CTC's from the normal cells based on the size. Then along with size deformability this characteristic can also be exploited.

So, what is that? That is CTC's are mostly larger in size. So, when during the filtration based enrichment the larger CTC's will be separated from other cells. Another technique is chip based separation where along with the this large CTC's they are rather stiff. So, the other cells which can pass through the chip, but because CTC's are not deformed they will be gathered or collected over the microchip. Again Ficoll gradient based density, Ficoll density gradient based separation based on the density gradient centrifugation method CTC's can be separated.

Dielectrophoresis another method of separating the CTC's by applying electrical electric charges. So, what happens under a particular medium conductivity different types of cells are basically directed to different electrodes. So, CTC's can be gathered or can be collected in a specific electrode. Apart from that single spiral microchannels are also used for separating CTC's. So, spiral CT chips are basically exploiting the CTC size.

So, what happens under the influence of deemed drag forces these are a physical pattern physical pattern for different cells. So, what happens smaller blood cells they will

migrate along the dean vertices towards the inner wall you can see here this is the inner wall and this is the outside wall. So, the smaller cell first will go towards the inner wall, but then they will be back on the outer wall again. Whereas, the larger cells which are the CTC's they experience one additional strong inertial lift forces. So, they will be attracted towards the micro channels inner wall.

So, this is how CTC's can be separated based on different physical properties which are different from other cells which are circulating in the blood vessel. Enrichment can also be done by exploiting a combination of both physical and biological properties. So, what happen initially there is enrichment following the size of the CTC's sorry following the size of the CTC's then after that based on their protein expression they can be enriched. So, what happens CTC's are first selected on the basis of their presumably larger size which separate it is from the smaller leukocytes after that they are specifically captured using a antibodies which is basically conjugated over a bead which contains antibody against epithelial cell adhesion molecules. So, what happens under the magnetic field those larger cells which are presumably CTC's now they are properly captured.

Even the opposite one can also be done the larger CTC's if they are having any contaminating leukocytes those leukocytes can be depleted by separating the leukocytes which contains the beads contain antibodies against CD45 and CD50 which actually marks the leukocytes. So, the combination of both physical and biological properties can be applied during enrichment. So, these are the different approaches for enrichment where immuno captured is one such method where based on the positive and negative enrichment where the expression of different markers are exploited. So, one such gold standard platform is cell search cell search where there is immuno magnetic enrichment using ferrofluid which is coated with anti epam antibodies in the device and there is auto immuno staining of the separated cells along with that there are also markers for cytokeratin's those cells which are isolated they are basically imaged via different fluorescent channel. So, basically one CTC is the CTC's are enriched based on both epithelial marker as well as cytokeratin markers also leukocyte marker negativity.

So, one proper CTC is basically defined as cytokeratin positive CD45 negative cell with an intact nucleus which has one DAPI positive staining must be round or oval in shape which has a diameter of at least 4 micron. But what is the drawback here the drawback is basically EMT or epithelial to mesenchymal transition. So, cells tumor cells which have undergone this transition they are basically now expression mesenchymal markers. So, they have less epithelial markers or epam or cytokeratin's. So, those cells are basically they are escaped from the system.

So, there are multiple other immunomagnetic capture methods which follow the

immunomagnetic negative enrichment also. Easy safe technology is another popular technology where magnetic field magnetic field is used to retain the leukocyte where the resulting supernatant basically it contains the heterogeneous population of CTC's. Now from that supernatant CTC's are basically collected. Again what is the drawback here the drawback here is the rare nature of CTC's. CTC's sometimes it can combine with the neutrophils.

So, what happens the neutrophilic markers are expressed over that this is how they can escape the easy safe technology techniques as well. There are different microfluidic devices microfluidic devices where different smaller channels are there over a platform through which the blood flow in a control speed and in those micro channel there are labels or surface markers the antibodies are captured which can capture the cell based on the surface marker or can exploit different physical properties like cell size. Some integrated nanomaterial based separation or enrichment of the CTC's can also be done. Apart from the biological and physical property based separation method there are different functional assays which are required to not only detect, but to characterize the tumor cells mainly to identify metastasis initiator cells or MIC's. So, the cells of tumor which are capable to metastasize they exhibit certain properties which can pass evade through the local tissue can enter and survive in the circulation and further it can extravacate to the distant foreign organs.

So, those among those functional assays two very common are epi spot assay and invasion assay. So, epi spot assay as you can see it basically detects specific proteins which are secreted from the tumor cells in in vitro culture and they are identified by fluorescent antibodies. Also invasion assay, invasion assay basically amongst the available functional assays one is epi spot assay. Epi spot detects the specific proteins which are secreted from the tumor cells in in vitro culture techniques and those secreted proteins those proteins are identified based on fluorescent antibody targeted towards those proteins. Similarly, invasion assay, invasion assay basically detects the invasion.

How they detect the ability of the CTC's to digest the extracellular matrix and those extracellular matrix proteins are basically labeled with fluorescent tag. So, these are the two available functional assays which can characterize the tumor cells whether they are having the metastatic characters for that what to be done? Initially the CTC's needs to be isolated using the previous method enrichment method after that those CTC's are applied for epi spot or invasion assay. These are the in vitro techniques. Some in vivo techniques are also exploited to check the characteristics of CTC's via transplanting those CTC's those CTC's are patient derived CTC's transplanting them in immuno deficient mice and check whether they are metastasizing or not. So, this is how different functional assays can be done to check the metastatic capability of a tumor cell.

So, this is the approach for the tumor cells cancer circulating tumor cells. So, after isolation immunostaining can be done different genomic techniques like RT-PCR sequencing in situ hybridization technique can be done different proteins analysis can be checked in in vitro and in vivo techniques they are metastasizing properties can be checked So, this is the pattern this is the protocol by which we can check the circulating tumor cells. Next, coming to circulating tumor DNAs. So, in circulation there are cell free DNAs. These are the fragmented DNAs which are found in body fluids which are released from different types of cells.

In tumor those fragmented DNA basically the fragmented DNA which are coming from the tumor cells they are known as CT or circulating tumor DNA and they are around 1 to 2 percent of all the cell free DNAs in the circulation. Now what is the purpose? The aim is to distinguish the circulating tumor DNA from other normal cell free DNA proteins. How that can be done by identifying certain epigenetic markers or genetic markers which differentiate those cell free tumor originated cell free DNA from the normal cell free DNAs. And this is how treatment protocols can be designed prognosis can be estimated some recurrence or residual disease can be identified. Now, where are these circulating tumor DNAs are coming from? Those circulating DNAs can come from tumors active secretion to the circulation.

When the tumor cells undergo apoptosis somehow the the DNAs can come in circulation also on necrosis the DNA can come into circulation. And they show certain characteristics certain genetic changes which we already have discussed in the previous class of tumor tumorogenesis that there can be point mutation which can be identified there can be methylation hypo or hyper methylation which can be identified. Copy number variation in term of micro satellite instability can be identified structural changes DNA damages different micro RNAs they all can be identified in the circulation. So, what is the approach after collection of the sample? The samples stored in minus 80 and transported and on that very day tumor DNA circulating tumor DNA needs to be assessed because they are very much unstable. So, what are the approaches like pyro sequencing can be done next generation sequencing can be done digital PCR which we are going to in the discuss in the next class that can also be done to detect different genetic markers distinguishing markers of the circulating DNA which can distinguish them from the normal cell free DNAs.

But of course, there are certain limitations in circulating tumor DNA assessment strategies like in the early stage of the cancer there is a very low concentration of the circulating DNA in the cell. And for that what is needed a very high blood volume is required to detect that very low concentration of the circulating tumor DNA. Then itself the cell free DNA the normal or the tumor DNA cell free DNA their half life is very variable from 1 minute to 2 and half hours in the system. So, basically when to target for

detection is yet not standardized. Of course, in the blood stream there are variable pattern of release.

So, basically it might not correlate with the concentration of the circulating tumor DNA might not correlate with the tumor stage and size. In all the metastatic diseases it is not always present only 75 percent patients they show positive for circulatory tumor DNA. And the I mean the concentration of the circulatory tumor DNA the they are not again standardized with the tumor anti cancer therapy and the responses. So, these are the limitation yet which is present in incorporating the liquid biopsy based on circulatory tumor DNAs. Then we are coming to another biomarker that is tumor educated platelets.

So, platelets they basically help in both local and systematic invasion of cancer or systematics or local spread of cancer. How what happens the platelets they basically interact with the tumor cells in such a way that even sometimes they bind and on during this process of interaction somehow the platelets are educated rather the tumor cells make them contribute in tumor progression and metastasis because they induce some altered behavioral pattern in the in those platelets. So, this is how platelets can help in metastasis. So, it induces certain growth factors induces certain angiogenesis factor then in intravascular phase when it is inside the circulation it offer protection from stress. So, the degradation of platelets are basically prohibited it even evades the immune response of the the immune response of the host or the patient then you can see secret or up regulate components of extracellular matrix.

So, this is how platelet can take part in spreading of tumor. Now how this education process is done basically during the interaction of this tumors tumor cells and platelet what happens there is alteration of the RNA profile of the tumor of the platelet. Remember platelet it does not contain nucleus, but it contains RNA micro RNA then messenger RNA, tRNA, rRNA, small nucleolar RNA, pre mRNA all these are present in the platelet and their expression profile is basically altered via sequestration of tumor specific biomolecules some tumor specific splice events occurs also mega cariocyte alteration the physiological properties sometimes changes. So, there is a there is one altered RNA profile in tumor educated platelet apart from that this platelet continuously exchange circulating nucleic acid and proteins via vesicles with the tumor and its micro environment and also the immune cell endothelial cell, stromal cell they also because they are also changing their expression pattern those cells they express some apart from this apart from this internal RNA profile alteration platelet also exchange circulatory nucleic acid and proteins via vesicle mediated transport from not only tumor cells, but also its micro environment where different immune cell endothelial cell and stromal cells are present. Now these cells immune cells endothelial cells stromal cells they are also changing their expression pattern.

So, those differentially expressed proteins or different biomarkers nucleic acids they can be transported to platelets and changes the platelets behavior. So, the platelets basically harbors and express certain tumor specific elements inside it. Now this education of tumor in TEPs or tumor educated platelets there can be a direct education that is transfer of specific tumor specific biomolecules or indirectly platelets are modified by a transcriptional splicing. So, what happens in transcript post transcriptional splicing external modification of platelet via tumor bone marrow communication can be done. So, direct transfer of certain biomaterials can be there or internally platelets can express differentially via post transcriptional splicing.

So, this is how tumor educated platelets helps in liquid biopsy which when is isolated from a patient the diagnosis of the cancer can be done via the differential expression, then classification of the cancer can be done also certain mutations can be done based on that the further diagnosis can proceed. Apart from this circulatory DNA circulatory tumor cell and tumor educated platelets certain other biomarkers are also detected in liquid biopsy like methylation markers via bisulfite sequencing which we have discussed already. Then extracellular vesicles in the form of exosome micro vesicles or apoptotic bodies they are basically taking part in normal physiological phenomena where they carry a cargo which contains the lipid protein RNA DNA fragment from the cells or from the origin where it has where from it has been secreted. So, basically there is a molecular fingerprint over the cell of origin. There are different proteins also which are expressed in tumor cells like prostate specific antigen, tumor they are mostly known as tumor markers like cancer antigen 125 CA 125 which is present in ovarian cancer, but the problem with these proteins are basically they are not specific neither they are sensitive.

Apart from the DNA nucleic acid like ribonucleic acid or RNA that can also help in diagnosis of the liquid biopsy because of course, the micro RNA expression RNA expression the cell free RNA which are secreted which is basically secreted from the tumor cells their expression patterns are different from the other normal cells. So, coming to the summary liquid biopsy is basically helping in analysis of tumor where the sample is definitely body fluids which can be CSF blood urine different tissue fluid saliva where thus common biomarkers are circulatory tumor cells circulating tumor DNA tumor educated platelets along with certain other biomarkers like methylation specific biomarkers, tumor markers as proteins circulate circulatory tumor RNA this can be detected in liquid biopsy. Circulatory tumor cells before assaying or detecting them we enrich them to increase the concentration based on certain biological or physical properties of those tumor cells. Then tumor educated platelets are there which exhibit altered RNA profile due to sequestration of tumor specific biomolecules or different post transcriptional splicing events and these platelets directly or indirectly they express tumor specific elements which basically distinguishes these platelets from the normal

circulating platelets.

So, these are my references. Thank you and see you in the next class.