Review

Identification of LncRNAs Associated Proteins Using Mass Spectrometry-Based Proteomics Landscape for Unlocking the Secrets of Cancer

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Review

Identification of LncRNAs Associated Proteins using Mass Spectrometry-Based Proteomics Landscape for Unlocking the Secrets of Cancer

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Abstract: Background/Objectives: To develop a novel method for isolating proteins associated with Long Non-coding RNAs (LncRNAs) using high-throughput Liquid Chromatography Mass Spectrometry (LC-MS) and proteomics approaches for identifying cancer-associated proteins involved in post-translational modifications (PTMs) such as glycosylation, phosphorylation, citrullination, methylation, and acetylation. Additionally, the focus is on identifying and quantifying differentially modulated or regulated proteins associated with LncRNAs to discover novel molecular signatures or biomarkers. **Methods**: Identification, quantification, and characterization of LncRNAs associated proteins by using Label free Quantification (LFQ) of proteins by Nono LC-Mass Spectrometry (LC-MS)., use Employ bioinformatics, biostatistics analysis for find out the differentially expressed, exclusive protein or modulated., find outInvestigate the mechanisms and pathways involving these unique or specific proteins analysis of unique/specific proteins and Validation of these unique/specific proteins by Immunohistochemistry (IHC) and western blotting/ enzyme-linked immunosorbent assay (ELISA) for to achieve better outcome. **Conclusions**: Identified and validated novel proteins associated with LncRNAs could be early detective molecular biomarkers of cancer and it could be useful in clinical practice for diagnosis, prognosis of liver, colorectal and other several types of cancer types.

Keywords: long non-coding RNAs (LncRNAs); protein and pathways analysis; liver and colorectal cancer; liquid chromatography mass spectrometry (LC-MS); novel molecular signature/biomarkers; diagnosis and prognosis

1. Introduction

Long non-coding RNAs (LncRNAs) represent a diverse and functionally significant class of RNA molecules that do not encode proteins but play critical roles in regulating gene expression and cellular functions [1]. The isolation, identification and quantification of proteins which are associated with lncRNAs are difficult to understand [2, 3]. Unlike proteins, which can often be studied using antibody-based techniques, lncRNAs require different investigative methods due to their distinct characteristics and interactions. LncRNAs can act as scaffolds to bring together various molecular components to form functional complexes essential for chromatin remodeling, transcription regulation, and RNA processing [4]. Their unique regulatory roles and involvement in diverse biological processes highlight the need for specialized techniques to study these molecules comprehensively, which begins with their identification and characterization. High-throughput sequencing technologies, such as RNA-seq, have been instrumental in identifying lncRNAs across different tissues and disease conditions, providing a comprehensive view of the transcriptome and revealing novel lncRNAs along with their expression patterns [5]. Bioinformatics tools predict the secondary structure of lncRNAs and identify potential binding sites for proteins, RNA, and DNA, while experimental techniques like RNA immunoprecipitation and crosslinking

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immunoprecipitation validate these interactions *in vivo*[6]. One such innovative approach involves pulling out lncRNAs using complementary oligonucleotide probes, which act as primers. These probes hybridize specifically the lncRNA of interest, allowing for its selective enrichment from a complex RNA mixture [7]. Once isolated, the lncRNA can be tagged with biotin, facilitating subsequent purification steps and enhancing the detection and analysis of the lncRNA and its associated proteins or other interacting molecules [8]. A second primer, complementary to a different region of the lncRNA, can further amplify or label the RNA, allowing for a highly specific and efficient way to study lncRNA interactions within the cell [9].These advanced techniques are particularly valuable in cancer research. Dysregulated lncRNAs are involved in regulating genes that control cell proliferation, apoptosis, metastasis, and help in identifying proteins that are hallmarks of cancer, such as proteins involved in oncogenic signaling pathways or tumor suppressor functions. One such example is lncRNA MALAT1, which interacts with proteins that promote metastasis in lung cancer [10]. Understanding these interactions is very crucial for developing these targeted therapies. By isolating and studying lncRNAs i.e., HOTAIR, researchers can identify chromatin-modifying complexes involved in cancer and design drugs that can disrupt these interactions to inhibit cancer-promoting effects [11]. The therapeutic potential of lncRNAs extends beyond cancer. LncRNAs are involved in various diseases, including cardiovascular diseases, neurological disorders, and metabolic diseases. For example, the lncRNA ANRIL is implicated in atherosclerosis and cardiovascular risk, while NEAT1 is associated with neurodegenerative diseases [1, 12]. Targeting these specific lncRNAs could provide a novel therapeutic strategy for these various conditions. Moreover, the specificity and distinct expression patterns of lncRNAs make them attractive candidates for non-invasive cancer biomarkers. For example, the lncRNA PCA3, specific to prostate cancer, can be detected in urine, providing a non-invasive diagnostic tool [13-15].High-throughput sequencing technologies and bioinformatics tools have advanced our understanding of lncRNAs' regulatory roles and interactions. Techniques like RIP, CLIP, and CRISPR/Cas9-mediated genome editing have enabled researchers to map the interactome of lncRNAs and study their molecular functions comprehensively [16]. These methods have revealed the essential roles of lncRNAs in development, differentiation, and various diseases.

In short, lncRNAs are critical regulators of gene expression and cellular functions, with unique roles distinguishing them from protein-coding genes. Advanced techniques for studying lncRNAs, such as oligonucleotide probes, biotin tagging, RIP, CLIP, and CRISPR/Cas9-mediated genome editing, have greatly enhanced our understanding of their biology. These techniques are particularly valuable in cancer research, where lncRNAs play crucial roles in oncogenesis and tumor progression. Identifying lncRNA-associated proteins and understanding their interactions can reveal novel therapeutic targets and biomarkers, which can help pave the way for innovative treatments for cancer and other diseases. In this article, we describe a new method for the isolation, identification, quantification, and characterization of lncRNA-associated proteins using high-throughput nano liquid chromatography-mass spectrometry (LC-MS). Additionally, we validate the identified differentially expressed or modulated proteins that could be exclusively associated with lncRNAs using commercially available antibodies through western blotting and immunohistochemistry (IHC)/enzyme-linked immunosorbent assay (ELISA). This approach aims to uncover novel molecular signatures that could be useful in the diagnosis and prognosis of liver, colorectal, and other associated liver cancers/diseases, such as chronic liver disease (CLD), acute-on-chronic liver failure (ACLF), nonalcoholic fatty liver disease (NAFLD), severe alcoholic hepatitis (SAH), and other human diseases[3, 17],[13, 15].

2. Method:

2.1. Isolation and Identification of LncRNAs Associated Proteins

The identification of LncRNAs associated proteins is crucial for the understanding of molecular mechanisms of their function and regulation[2, 18]. The RNA Antisense Purification with Mass Spectrometry (RAP-MS) technique revolutionizes our ability to pinpoint direct RNA-protein interactions within living cells[19]. This method provides a detailed roadmap for isolating proteins

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directly associated with lncRNAs[19]. Whether utilizing whole-cell lysate or nuclear extract, the process culminates in a comprehensive roster of proteins intricately engaged with the target RNA within the dynamic milieu of cellular environments. Central to this method is the strategic design of 90-nucleotide oligos, engineered to cover the entire sequence of the lncRNA, with careful consideration given to avoid off-target effects through LC-MS analysis of the data. The incorporation of a 5′ biotin standard modification facilitates the efficient pull-down of lncRNA-associated proteins. Other studies, such as those focusing on isolating protein complexes associated with lncRNAs from HeLa cells, underscore the versatility and applicability of this approach[20].

Furthermore, advancements such as the HyPR-MS method [21] have enhanced the efficiency and scope of RNA-protein interactome analysis. By leveraging predictive RNA secondary structure modeling through M-fold software, HyPR-MS streamlines capture oligonucleotide design, optimizing experimental outcomes while minimizing design time [21, 22]. Expanding the horizon of RNA interrogation, Engreitz et al. introduced RNA antisense purification to map RNA-RNA interactions (RAP-RNA), providing systematic means to enrich specific RNAs and identify interacting molecules [23]. This technique, exemplified in the study of xist lncRNA localization during X-chromosome inactivation [24], offers invaluable insights into lncRNA-mediated chromatin regulation[24]. Complementary methods such as Chromatin Isolation by RNA Purification (ChIRP) and Capture Hybridization Analysis of RNA Targets (CHART) provide high- throughput avenues for elucidating RNA-bound proteins and genomic binding sites of specific lncRNAs [25]. A pool of short complementary DNA oligonucleotide probes inspired by RNA FISH, CHART adapts an RNase H mapping assay, offering nuanced approaches tailored to different experimental contexts. Additionally, Reversible Cross-Linked Immune Precipitation (ReCLIP) emerges as a powerful tool [26, 27], preserving loose protein associations intact to identify lncRNA-associated proteins. By leveraging cell-permeable, thiol-cleavable crosslinkers and in-cell crosslinking, ReCLIP captures endogenous protein-protein interactions with remarkable fidelity, offering a glimpse into the dynamic landscape of RNA-protein interactions within living cells[25, 27]. Interaction with protein complexes is a common mechanism for the functions of lncRNAs. Thus, identifying proteins associated with lncRNAs is critical for the understanding of molecular mechanisms and functions of lncRNAs. Immunoprecipitation is commonly used for the isolation of protein complexes associated with a protein of interest. However, this method is not available for lncRNAs because antibodies generally do not recognize RNAs.[28, 29] [30] Proteins that have higher MS counts in cells transfected with lncRNA-MS2 than controlled MS2 plasmid are selected as potential candidates. Knockdown of these protein candidates with short hairpin RNAs (shRNAs) is used to confirm whether any of the candidates are required for the functions of lncRNA of interest. Further validation of the binding of protein candidates to lncRNA of interest is required to confirm their association [2, 3].

2.2. Identified Pathways Associated with lncRNAs:

LncRNAs associated proteins are emerging as significant players in the diagnosis and therapy of various cancers [27]. The extensive discovery and reporting of lncRNA-associated proteins highlight their diverse expression patterns and tumor-specificity across different cancer types [22, 24, 29]. Table 1 lists several key lncRNA-associated proteins such as MALAT1, UCA1, HULC, HOTTIP, CCAT1, CCAT2, and H19, detailing various methods used for their isolation and identification, which serve as biomarkers for treating liver and colorectal cancer patients [31-34]. These proteins also facilitate further mechanistic and pathway studies of lncRNAs, enhancing our understanding of cancer pathophysiology.

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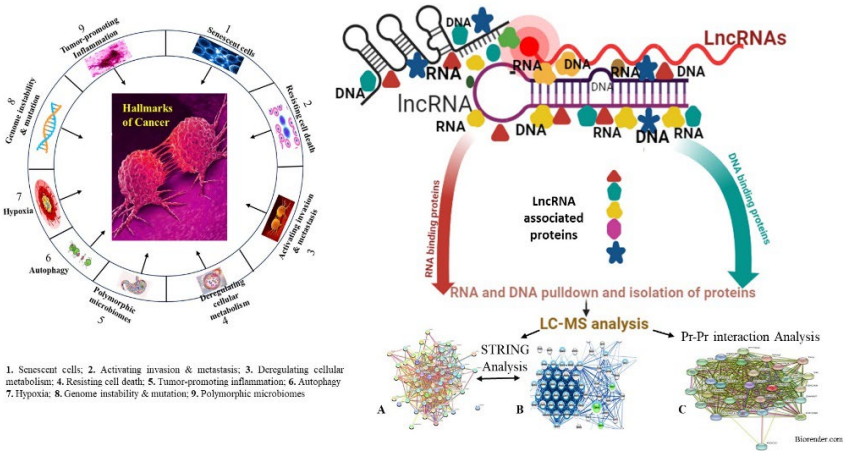

Figure 1. Hallmarks of Cancerof and Long Non-coding RNAs (LncRNAs), How the identification of associated protein identify, quantification, and characterization by LC- Mass Spectrometry (LC-MS). And this is the flow work chart of differentially expressed significant proteins with bioinformatics of the novel molecules.

Table 1. Identified lncRNA-associated proteins and utilized methods: The table includes serial numbers, study groups, lncRNA-associated proteins, methods used, and remarks/conclusions on these proteins and their further use in liver, colorectal, and other types of cancer.

S No.	Study	Target LncRNAs	Specific Method name (Cancer CRC & HCC)	Remarks/conclusions	Reference
1	Shijian Fu et al	MALAT-1	Patient samples & ovarian cancer cell lines (SKOV3 & CAOV3)	MALAT-1 diagnostic or prognostic biomarker or therapeutic target in the treatment of many cancers.	**[35]**
2	An Yang et al	UCA1	Via the miR-145/MYO6 axis	The UCA1/miR-145/MYO6 axis may serve as a potential therapeutic target for gastric cancer.	**[36]**
3	Enans M et al	T2D/ HCC	NAFLD/T2D-associated HCC	Metformin may reduce the risk of cancer in patients with T2D. The unadjusted odds ratio was 0.86 (95% CI 0.73 to 1.02). The unadjusted odds ratio for any exposure to metformin since 1993 was 0.79 (0.67 to 0.93)	**[13, 37]**
4	Fabrizio Ferre et al	Revealing protein	RAPID-SELEX RNAcompete RNA Bin-n-Seq RNA-Ma	Better understanding of lncRNA cellular mechanisms and their disease-associated perturbations.	**[37, 38]**
5	Fabrizio Ferre et al	LncRNA interactio n	MS2 trapping SILAC-based Phage display Protein arrays	LncRNA-bound proteome, or if still uncharacterized protein domains and architectures are involved, network will be high	**[37]**
6	Arunoda y Bhan et al	HULC	Tumorigenesis test in vitro and in vivo: RT-PCR, W. B	Potential implications in cancer diagnosis and therapy	**[33, 37]**
7	Chunqin g Wang et al	HULC	HULC interacts with the glycolytic enzyme LDHA	HULC promotes Warburg effect by orchestrating the enzymatic activities of glycolytic enzymes	**[34, 39]**
8	Chunlin Ou et al	Linc00152	Human Tissue Samples:	Targeting YAP1/LINC00152/FSCN1 Signaling Axis Prevents the Progression of Colorectal Cancer	**[40]**
9	Jie-yu Sun et al	HEIH	Non-coding RNAs	Nearly 8000 cancerspecifc lncRNAs have been nominated, PCA3 is prostate-specific, prognostic biomarker prostate cancer.	**[41]**

 doi:10.20944/preprints202410.2189.v1

10	Huaili Jiang et al	HOTTIP	In silico analysis, Plasmid construction and transfection	Significantly, M1 exosomes and HOTTIP polarize circulating monocytes into the antitumor M1 phenotype, which may provide novel insight into HNSCC immunotherapy.	**[42]**
11	Taruna Rajagopal et al	HOTAIR	HOTAIR mediated gene silencing	It could be used in conjunction with current drugs to sensitize tumors to the existing therapies	**[43]**
12	Yang Mu et al	CCAT1	RT-qPCR to level of miR-490-3p and CCAT1	facilitate developing novel therapeutical therapies for treating ovarian cancer.	**[44]**
13	Peng Gao et al	CCAT2	Dimethyl sulfoxide (DMSO) (Aldrich, St. Louis,	to explore genes co-expressed with lncRNA CCAT2 and functional molecular	**[45]**
14	Hashemi et al	H19	Enhancing growth and cell cycle of cancers and by EMT induction	Increased proliferation Glycolysis induction miRNA-519d-3p down-regulation by H19 to increase LDHA expression	**[46]**
15	Hua Fang et al	CCAT1-L	Quantitative real-time PCR and Western blot, respectively.	inhibits epithelial–mesenchymal transition of gastric adenocarcinoma cells and thus suppresses the gastric adenocarcinoma metastasis.	**[47]**
16	Feifei Zhang1	CRNDE	Chemosensitivity of GC in clinical samples and a PDX model.	Highlighting the significance of CRNDE as a potential prognostic marker and therapeutic target against chemoresistance in GC.	**[48]**
17	Yanping Li et al	FER1L4	Cell was extracted from embryos of rat	FER1L4 modulates the proliferation and differentiation of NSCs via regulating Ascl2.	**[28]**
18	Yingrui Fan et al	PTENP	uciferase reporter assay and RNA-pulldown assay	Inhibit cell proliferation and EMT and induce cell apoptosisin cervical cancer cells.	**[49]**
19	Jinmai Jiang et al	T-UCRs	qPCR array to profile all 481 T-UCRs in pancreatic cancer specimens, pancreatic cancer cell lines	Expression of T-UCRs in both human and mouse PDAC and similar mechanism of upregulation in PDAC	**[50]**
20	Arunoday Bhan et al & John H Boyd	TUC338	Plasma, treatment, and cell lines, MS2-MBP Protein Expression and Immobilization	The understanding of molecular mechanisms of lncRNAs. Inhibition of PCSK9 activity is an attractive target for treating the spectrum of sepsis and septic shock.	**[33, 51]**

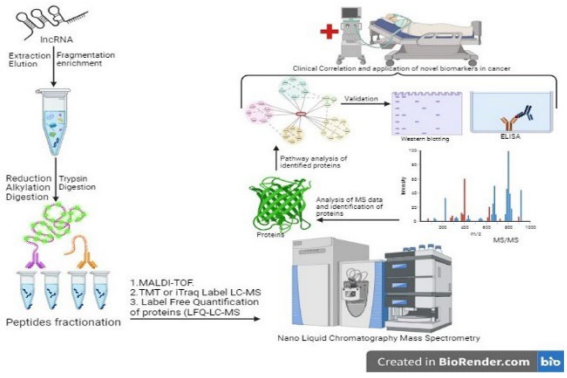

Figure 2. Isolation of Long Non-coding RNAs (LncRNAs) associated protein then sample preparation for identification, quantification, and characterization of isolated/eluted protein by LC- Mass Spectrometry (LC-MS). Analysis of all MS data for identification of differentially expressed significant proteins after the validation of those proteins by Western Blotting and Immunohistochemistry (IHC)

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or the enzyme-linked immunosorbent assay (ELISA). Clinical correlation of identified and validated proteins that could be early detection biomarkers for diagnosis and prognosis of liver, colorectal and several types of cancer.

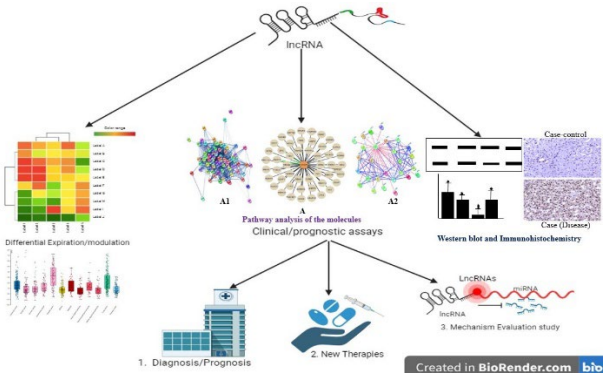

Figure 3. Bioinformatics, Biostatistics and Regulatory analysis of all LC-MS data and find the novel LncRNAs associated proteins which are clinically relevant for the liver and colorectal cancer mechanisms or pathway that proteins will be validated by Western Blotting and Immunohistochemistry (IHC)or the enzyme-linked immunosorbent assay (ELISA). After validation of the above proteomics method, novel LncRNAs associated proteins could be helpful in early detection biomarker for diagnosis/prognosis, new therapies of anti-cancer drugs and further mechanistic study of liver, colorectal and other types of cancer.

A study by [52] focused on Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT-1) and its potential role as a diagnostic or prognostic biomarker for ovarian cancer. MALAT-1 expression was found to be significantly upregulated in ovarian cancer tissues compared to normal tissues. The findings suggest that MALAT-1 could serve as a therapeutic target due to its involvement in cancer progression, highlighting its importance in tumor growth and metastasis [29]. Explored Urothelial Cancer Associated 1 (UCA1) in gastric cancer, demonstrating that UCA1 interacts with miR-145 and MYO6, forming a regulatory axis that affects cancer cell proliferation and apoptosis, suggesting new therapeutic approaches targeting regulatory axis[53]. Examined the impact of metformin on cancer risk in type 2 diabetes patients, suggesting that metformin might reduce cancer risk, and highlighting its potential as a chemo-preventive agent and utilized techniques such as RAPID-SELEX and RNA compete to map lncRNA-protein interactions, providing insights into lncRNA cellular mechanisms and their disease-related perturbations, and further used MS2 trapping and SILAC-based display to identify lncRNA-bound proteomes, aiding in understanding lncRNA functions in cellular networks[54]. Another study focused on HULC, demonstrating its overexpression in various cancers and promoting tumor growth, suggesting its potential as a biomarker and therapeutic target[55]. Showed that HULC interacts with LDHA, promoting glycolysis in cancer cells, highlighting HULC's role in cancer metabolism[56]. Identified nearly 8000 cancer-specific lncRNAs, including PCA3 for prostate cancer, emphasizing their diagnostic and prognostic potential. A study suggested that M1 exosomes and HOTTIP polarize monocytes into an antitumor phenotype, suggesting a novel approach for immunotherapy[42] demonstrated that HOTAIR mediates gene silencing and enhances tumor progression, suggesting its use alongside existing therapies to sensitize tumors[43] and Another study showed that CCAT1 regulates miR-490-3p in ovarian cancer, indicating new therapeutic strategies [57]and the explored CCAT2's co-expressed genes, suggesting targeting CCAT2 pathways for cancer therapy. That found H19 enhances cancer cell proliferation and glycolysis by downregulating miRNA-519d-3p and upregulating LDHA, highlighting its potential as a therapeutic target[58] and its demonstrated that CCAT1-L inhibits EMT in gastric adenocarcinoma cells, suggesting its potential to prevent cancer metastasis. The identified CRNDE as a marker and therapeutic target against chemoresistance in gastric cancer [59], showed that FER1L4 regulates neural stem cell proliferation and differentiation, suggesting its therapeutic potential in neurodevelopmental disorders. Finally, it was demonstrated that PTENP inhibits cell

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proliferation and EMT while inducing apoptosis in cervical cancer cells, highlighting its potential as a therapeutic target as well[49]. These brief explanations of various studies are examples of LncRNAs associated proteins, and their further use in liver, colorectal and others several different types of cancer.

3. Discussion:

Liver and colorectal cancer are the third most common causes of cancer-related deaths. These cancers are highly aggressive and resistant to treatment. In liver cancer, including hepatocellular carcinoma (HCC), which accounts for 75% to 85% of cases, and intrahepatic cholangiocarcinoma, which makes up 10% to 15% of cases, shows the highest mortality rates in Asia and Africa. However, incidence and mortality rates are also increasing in Europe and the US [17, 28, 60]. Chronic liver disease (CLD), Acute-on-chronic liver failure (ACLF), Nonalcoholic fatty liver disease (NAFLD), severe alcoholic hepatitis (SAH), Drug and alcohol-induced liver injury and other human diseases promote liver and colorectal inflammation and increase oxidative stress, which accelerates oxidative cell death and promotes HCC. The HCC shares common altered metabolic pathways with liver cirrhosis, CLD, NAFLD and SAH suggesting the involvement of tumorigenesis promotion in dysregulated lipidaemia [34, 38]. The identified lncRNA-associated proteins are involved in molecular pathways related to cancer pathogenesis and are directly implicated in liver inflammation and tumorigenesis, though their specific roles are not yet fully understood. But these could be involved in the post-transcriptional regulation of gene expression processes, molecules finding or signatures widely proposed as candidate biomarkers for diagnosis and prognosis of cancer including liver and colorectal cancer [61-63]. In this study, we highlight some of the emerging key players among lncRNA-associated proteins involved in liver and colorectal cancer, as well as other types of cancer. These proteins play specific roles in diagnosis and prognosis, potentially aiding in outcome prediction and treatment of cancer and we specially focus on HCC and CRC patients. [18, 29].

4. Conclusions and Future Perspectives

We present this protocol for the isolation, identification, and characterization of LncRNAs associated proteins via LC-MS analysis, as there is no fixed method to their isolation and identification. This protocol includes the use of specific instrumentation software for LC-MS data analysis to identify novel molecular signatures or unique proteins related to liver and colorectal cancer, which could play a significant role in disease progression with clinical correlations [64].

This process is shown in Figure 1, Figure 2 and Figure 3, which illustrate the validation of lncRNA-associated proteins. Validation is crucial for confirming the relevance of identified molecules and their correlation with disease pathways, aiding in outcome prediction for various type cancers mainly liver and colorectal cancer. This information is vital for understanding the relationships between single genes and multiple proteins, single proteins and many amino acids, and how all these elements correlate within polypeptide chains [17, 65].

The future of Reversible Cross-Linked Immune Precipitation (ReCLIP) in LncRNA research is relevant to significantly advance our understanding of RNA-protein interactions. Integrating ReCLIP with high-throughput LC-MS analysis will enable comprehensive mapping of the lncRNA-protein interactome, uncovering novel protein partners and their binding dynamics. This can help in the development of targeted interventions, such as small molecules or CRISPR-based therapies, to disrupt critical lncRNA-protein interactions involved in cancer and other diseases. ReCLIP's application in neurological, metabolic, cardiovascular, and immune-related disorders could lead to exclusive novel biomarkers and therapeutic targets.

Author Contributions: MSH initiated the writing of the manuscript, MKT given the idea about LncRNAs associated proteins. MSH made all figures and table, and MKT reviewed for improvement. PV help in written, drafted the manuscript with MSH and MKT. This manuscript has been seen and approved by all authors. Disclosure: All authors have declared no conflict of interest.

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