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MINCR: A long non-coding RNA shared between cancer and neurodegeneration

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ABSTRACT

The multitasking nature of lncRNAs allows them to play a central role in both physiological and pathological conditions. Often the same lncRNA can participate in different diseases. Specifically, the MYC-induced Long non-Coding RNA MINCR is upregulated in various cancer types, while downregulated in Amyotrophic Lateral Sclerosis patients. Therefore, this work aims to investigate MINCR potential mechanisms of action and its implications in cancer and neurodegeneration in relation to its expression levels in SH-SY5Y cells through RNA-sequencing approach. Our results show that MINCR overexpression causes massive alterations in cancer-related genes, leading to disruption in many fundamental processes, such as cell cycle and growth factor signaling. On the contrary, MINCR downregulation influences a small number of genes involved in different neurodegenerative disorders, mostly concerning RNA metabolism and inflammation. Thus, understanding the cause and functional consequences of MINCR deregulation gives important insights on potential pathogenetic mechanisms both in cancer and in neurodegeneration.

1. Introduction

Long non-coding RNAs (lncRNAs) are defined as transcripts of more than 200 nucleotides that carry out a plethora of functions, including transcriptional regulation, organization of nuclear domains, and stabilization of proteins or RNA molecules [11]. The mechanisms through which lncRNAs exert their functions are numerous and involve every aspect of cell life [35]. Moreover, the same lncRNA can act in multiple ways leading to different outcomes. Indeed, a single lncRNA can participate in the pathogenesis of more than one disease, even if these seem very different, as are cancer and neurodegenerative disorders, activating specific pathways which lead to one or another clinical phenotype. Although the outcomes in these diseases are very divergent, pathways involved in cellular proliferation and loss of cellular differentiation in cancer and progressive neuronal cell death in neurodegeneration can be the same but under a different modulation [8]. Mutations in a variety of genes involved in regulation of the cell cycle, DNA repair pathways, protein turnover, oxidative stress, and autophagy have been implicated in both clinical phenotypes [24]. Among the mechanisms affected, alterations in RNA metabolism are obtaining significant attention given the critical role of RNA transcription, maturation, transport, stability, degradation and translation in cellular

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functions [8] [15]. As a matter of fact, some of the most known lncRNAs, such as NEAT1 [39] [12], HOTAIR [18] [53] and MALAT1 participate in both clinical phenotypes where they exert different functions and, thus, lead to different outcomes. For instance, MALAT1 is upregulated in many types of cancer where it drives tumor progression through regulating tumor cell proliferation, metastasis and migration [19]. It is upregulated in myocardial infarction [59], it exerts a protective role in ischemic stroke [56] and it participates also in Parkinson's Disease where it increases the stability of α-Synuclein [57].

Precisely in this context, the lncRNA MYC-Induced Long non-coding RNA (MINCR) is emerging as a new potential regulation factor in different diseases. The MINCR gene is an intergenic lncRNA located between two coding genes, GLI4 and ZNF696, on chromosome 8q24.3. At least six different isoforms transcribed from the MINCR gene locus are annotated on ENCODE, the longest isoform composed of three exons and all others containing two exons. MINCR has firstly been described in

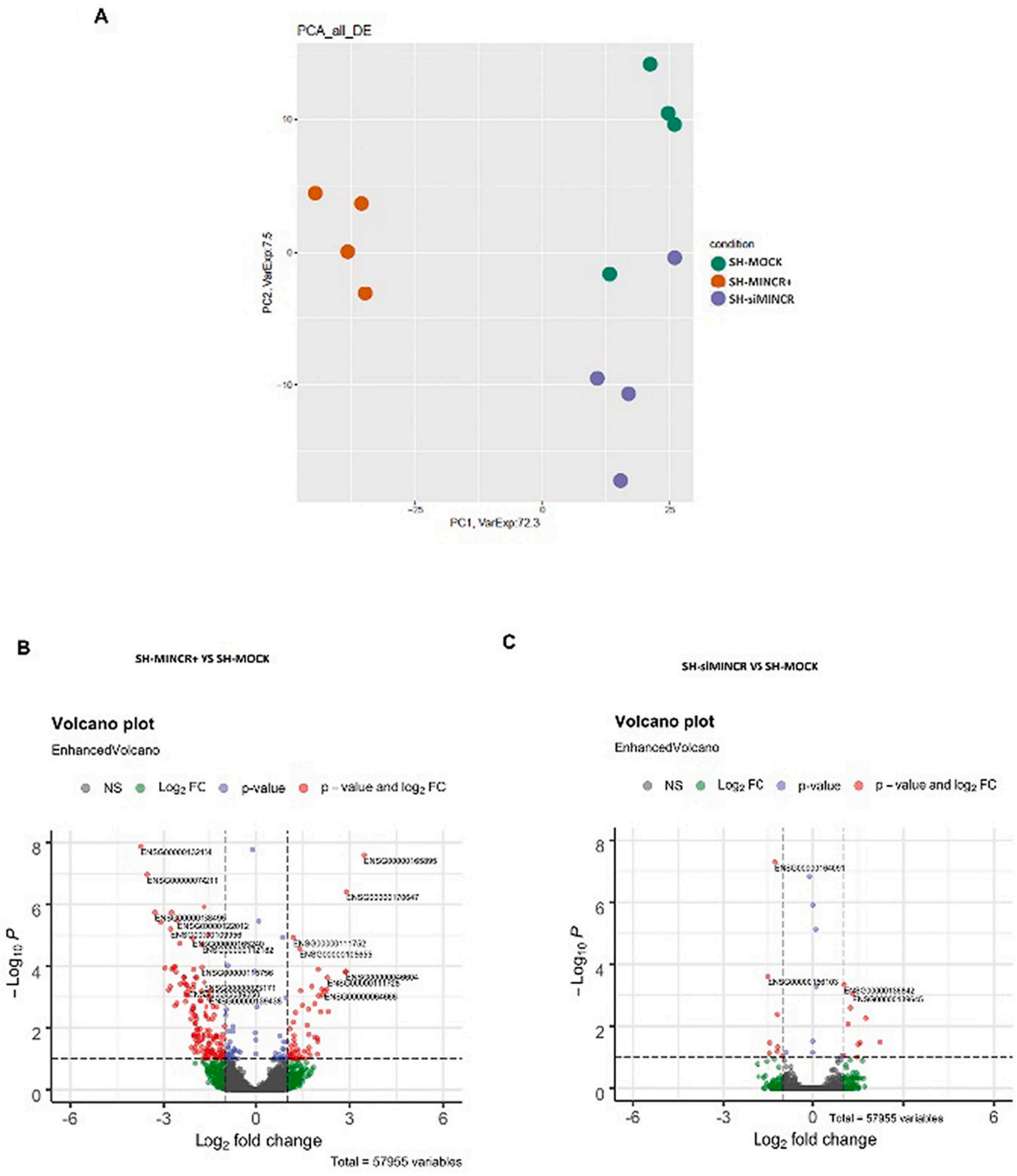

Fig. 1. SH-MINCR+ and SH-siMINCR show different RNAs expression profiles. (A): PCA of differentially expressed genes. All comparisons are given between the SH-MINCR+ and SH-siMINCR compared to SH-MOCK cells. (B): volcano plots of differentially expressed genes between SH-MINCR+ and SH-MOCK. (C): volcano plots of differentially expressed genes between SH-siMINCR and SH-MOCK. Ensemble Gene ID of the most deregulated transcripts are reported, red dots represent differentially expressed genes based on *p*-value and Fold Change. We considered as differentially expressed only genes showing $|\log2(\text{SH-MINCR+ or SH-siMINCR/SH-MOCK})| \geq 1$ and a False Discovery Rate (FDR) ≤ 0.1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MYC-positive lymphoma samples, where it showed a high positive correlation with MYC mRNA expression level and where its promoter region is bound by MYC protein at the transcription start site [13]. It has been also identified a positive significant correlation between MINCR and MYC mRNA expression in RNA-seq data from pancreatic ductal adenocarcinomas [13], where lncRNA MINCR knockdown experiments demonstrated that it is able to control the expression level of a set of MYC target genes involved in cell cycle initiation and tumor progression [13]. Moreover, MINCR is implied in other types of cancer, such as gallbladder cancer [54], hepatocellular carcinoma [33], non-small-cell lung cancer [10], oral squamous cell carcinoma [34], nasopharyngeal carcinoma [60] and glioma [32]. In all these tumors MINCR is overexpressed and correlates with poor prognosis, but its mechanism of action remains unclear. MINCR could be a key factor not only in cancer, but also in neurodegenerative diseases, considering its down-regulation in Peripheral Blood Mononuclear Cells of Amyotrophic Lateral Sclerosis (ALS) patients [14]. Moreover, rs7388117 MINCR variant, of uncertain clinical significance, has been reported in temporal cortex and cerebellum of Alzheimer's disease (AD) patients [61]. Therefore, the aim of this work was to establish the possible different mechanisms exerted by MINCR depending on its expression level in a neuronal context to understand its implications in brain cancer and neurodegenerative diseases.

2. Results

2.1. Deep sequencing RNA expression profiles in SH-MINCR+ and SH-siMINCR versus control conditions

To investigate the genes and the pathways through which MINCR exerts its effects, we performed a whole transcriptome analysis of SH-SY5Y neuronal cells carrying overexpression (SH-MINCR+) and down-regulation (SH-siMINCR) of MINCR compared to wild type cells (SH-MOCK).

We firstly characterized the model checking MINCR levels and its sub-cellular localization, showing that MINCR mostly localized in the nuclear compartment, although we detected its presence also in the cytoplasmic fraction (Supplementary Fig. 1A–1B).

Principal Component Analysis (PCA) obtained using differentially expressed genes (DEGs) dataset in SH-MINCR+ and SH-siMINCR compared to SH-MOCK shows different expression profiles, highlighting a larger difference between SH-MINCR+ and SH-MOCK (Fig. 1A). To avoid data manipulation and insert bias in our further observations, we included all the samples in the following analysis, although one of the SH-MOCK sample is nearer to SH-siMINCR than the others, probably because its MINCR expression levels are lower (RQ of 0.0025 compared to a mean of 0.0044 in qPCR). The two volcano plots (Fig. 1B) show the most significant DEGs in SH-MINCR+ and SH-siMINCR compared to SH-MOCK, confirming the different degree of alteration in the two cellular models.

Indeed, we detected a big difference between the number of DEGs in SH-MINCR+ and SH-siMINCR vs SH-MOCK, respectively. A total of 227 DEGs were identified in SH-MINCR+, while in SH-siMINCR we obtained alterations in 29 genes (Table 1, Supplementary Table 1 for full list).

Table 1
Number of statistically significant differentially expressed mRNAs and lncRNAs in SH-MINCR+ and SH-siMINCR compared to SH-MOCK, in terms of upregulated, downregulated and total deregulated transcripts. Transcripts were considered as differentially expressed when $|\log2(\text{SH-MINCR+ or SH-siMINCR/SH-MOCK})| \geq 1$ and a FDR ≤ 0.1.

	SH-MINCR+		SH-siMINCR	
	mRNAs	lncRNAs	mRNAs	lncRNAs
Upregulated	57	12	16	1
Downregulated	142	16	10	2
Sub-Total	199	28	26	3
Total	227		29	

Between the DEGs, the 2 most upregulated genes, in terms of fold change, are shared between SH-MINCR+ and SH-siMINCR, and these are ICAM1 and HMCN1. Both these genes are involved in cellular communication and migration, specifically ICAM1 is a transmembrane protein that stabilizes cell-cell interactions and facilitates leukocyte endothelial transmigration [55] and HMCN1 is an extracellular matrix protein that assembles into fine tracks to organize cell attachment into oriented linear junctions, such as hemidesmosome structure [51]. In addition to ICAM1 and HMCN1, 8 more genes are shared between the two cellular models (Supplementary Table 1).

2.1.1. MINCR's activity in sub-cellular compartments defined by DEGs protein distribution

Through "The Human Protein Atlas (Cell) - Protein Sub-cellular Localization" network of the Network Data Exchange (NDEx) software [38,48], we determined the subcellular distribution of proteins derived from deregulated coding genes in SH-MINCR+ and SH-siMINCR. In both cases the most implicated cellular compartment is the nucleus (Fig. 2A–2B). In SH-MINCR+ 54% of DEGs produce proteins localized in the nucleus, being part of one or more of the following structures: "Nuclear bodies", "Nuclear speckles", "Nucleoplasm", "Nuclear membrane", "Nucleoli", "Nucleus" and "Nucleoli fibrillar center". The remaining genes are distributed between the other cellular regions, such as "Cytosol", "Plasma membrane" and "Mitochondria" (Fig. 2A). We found similar results for SH-siMINCR, where the genes localized in "Nucleus", "Nucleoli", "Nucleoplasm", "Nucleoli fibrillar center" and "Nuclear bodies" are 48% of DEGs (Fig. 2B). Therefore, this analysis shows an enrichment of nuclear proteins as mainly dysregulated by MINCR changes in expression. Although siRNA silencing mostly affects cytoplasmic fraction of MINCR, it seems to have impact on every part of cell, even into the nucleus, as shown by protein sub-cellular localization analysis. This could suggest that MINCR activity inside the nucleus may be more sensitive to total expression level changes.

2.1.2. GO terms enrichment of deregulated coding transcripts

Coding gene expression profiles of SH-MINCR+ and SH-siMINCR versus control SH-MOCK were analyzed for Gene Ontology (GO) terms enrichment in Biological Processes, Molecular Function, and Cellular Component using EnrichR web tool [28] (Fig. 3).

When considering the top 5 GO terms for SH-MINCR+, in Biological Process and in Cellular Component we found terms such as "Nervous system development", "Neuron projection morphogenesis", "Axonal initial segment" and "Main axon" that mainly involved genes of neuronal processes, e.g., SHANK1 and SHANK2, which emerge as strongly downregulated (Fig. 3A-3B, Supplementary Table 2 for full list). Moreover, in Cellular Component we found a deregulation in "Integral component of plasma membrane" and "Focal adhesion". The GO Molecular Function highlights a deregulation in channel and receptor activity (Fig. 3C, Supplementary Table 2 for full list).

On the other hand, the top 5 GO terms in Biological Process in SH-siMINCR highlights terms related to protein structure and extracellular matrix organization (Fig. 3D, Supplementary Table 3 for full list). With respect to Cellular Component (Fig. 3E, Supplementary Table 3 for full list), we found deregulated terms concerning mRNA metabolism involving an epigenetic regulatory complex ("Set1C/COMPASS complex") and the "mRNA cleavage and polyadenylation specificity factor complex" which is a fundamental process in mRNA maturation. The other important alteration concerns the upregulated cytoskeletal remodeling genes, suggesting an altered cytoskeletal modification. We found cytoskeletal abnormalities also in GO Molecular Function, marking again the alteration in this structure (Fig. 3F, Supplementary Table 3).

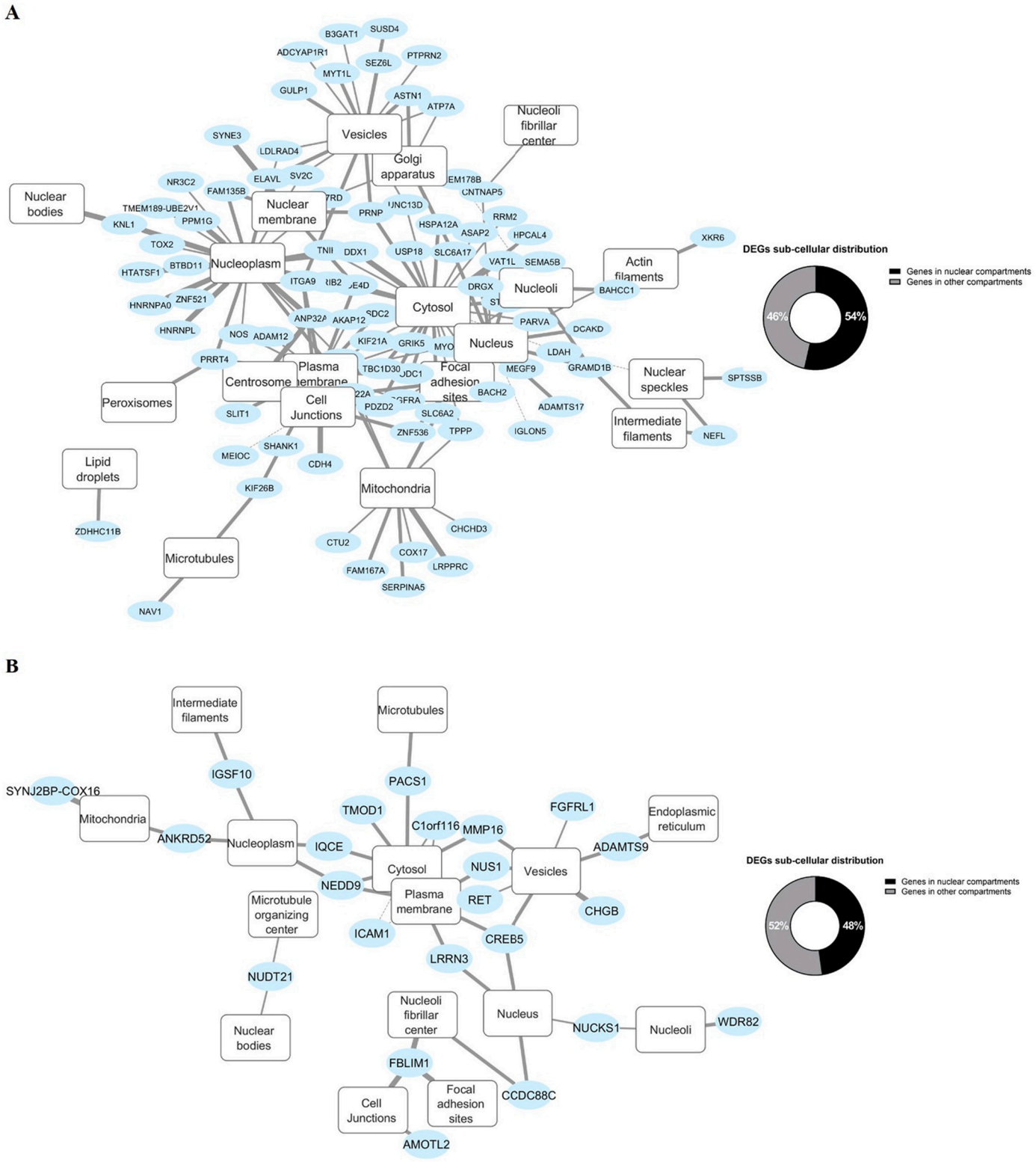

Fig. 2. Sub-cellular localization of differentially expressed coding genes in relation to MINCR levels. (A) Distribution of protein derived from DEGs in SH-MINCR+ and (B) in SH-siMINCR from "The Human Protein Atlas (Cell) - Protein Sub-cellular Localization" network of NDEx is shown.

2.1.3. Pathway analysis of deregulated coding transcripts

The deregulated coding transcripts with a deregulation ≥1 in terms of |Log2FC| were subjected to pathways analysis via the g:Profiler [41] and the EnrichR web tool [28]. G:Profiler analysis identified 105 significative enriched KEGG pathways in SH-MINCR+ and 51 in SH-siMINCR (Fig. 4A–4B) (full list of deregulated pathways is reported in Supplementary Table 4 for SH-MINCR+ and Supplementary Table 5 for SH-siMINCR). We firstly identified the top 10 deregulated pathways divided in up- and downregulated terms (Fig. 4C–4E for SH-MINCR+ and 4D–4F for SH-siMINCR). According to literature, the overexpression of MINCR is implicated in pathways involved in tumor onset and progression. This is particularly visible in the top 10 of upregulated

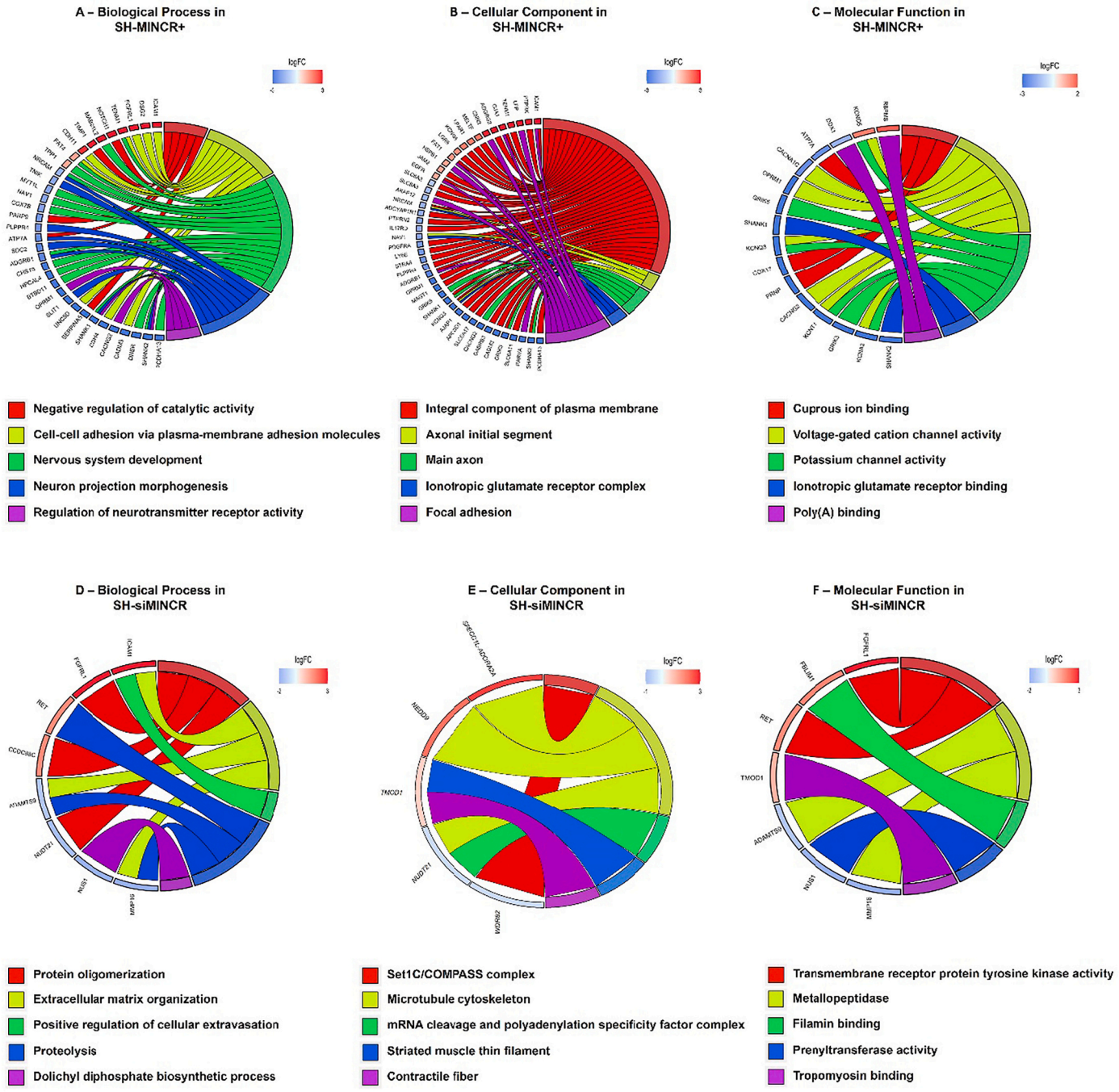

Fig. 3. Gene Ontology analysis for coding DE genes in SH-MINCR+ and SH-siMINCR compared to SH-MOCK. Top 5 enriched GO terms for (A) Biological Processes, (B) Cellular Component and (C) Molecular Function in SH-MINCR+ and for (D) Biological Process, (E) Cellular Component and (F) Molecular Function in SH-siMINCR are shown. Color represents different pathways connected to respective deregulated genes.

pathways in SH-MINCR+ samples, where the most significative term is "prostate cancer" in addition to other 2 cancer-related pathways (Fig. 4C). Neuronal pathways emerged as enriched in top 10 down-regulated terms in SH-MINCR+ (Fig. 4E), as GO analysis has already reported. A completely different pathways enrichment emerged in SH-siMINCR samples: the upregulated pathways are almost all related to inflammation (Fig. 4D), while the downregulated ones showed "mRNA surveillance pathway" as the most significative term (Fig. 4F). Interestingly, a cancer-related pathway appeared also in SH-siMINCR analysis ("microRNAs in cancer), but it is downregulated. The KEGG and WikiPathways analysis from EnrichR tool were consistent with g:Profiler results. Among the top 20 KEGG deregulated pathways in SH-MINCR+ (Fig. 4G), we found 5 pathways implicated in cancer mechanisms ("Prostate cancer", "p53 signaling pathway", "Melanoma", "Glioma" and "Bladder cancer"). WikiPathways analysis also showed tumor implication: "Pathways regulating Hippo signaling", "Hippo-Merlin signaling dysregulation" and "Deregulation of Rab and Rab effector genes in bladder cancer" (Supplementary Table 6). A full list of the 160 deregulated KEGG 2019 and of the 178 deregulated WikiPathways analysis for SH-MINCR+ is reported in Supplementary Table 6. In SH-siMINCR the emerging pathways from KEGG, containing at least 2 genes, were pathways concerning RNA metabolism and inflammation (Fig. 4H). WikiPathways analysis reported mRNA processing deregulation and a more considerable implication of inflammation, since 8 out of

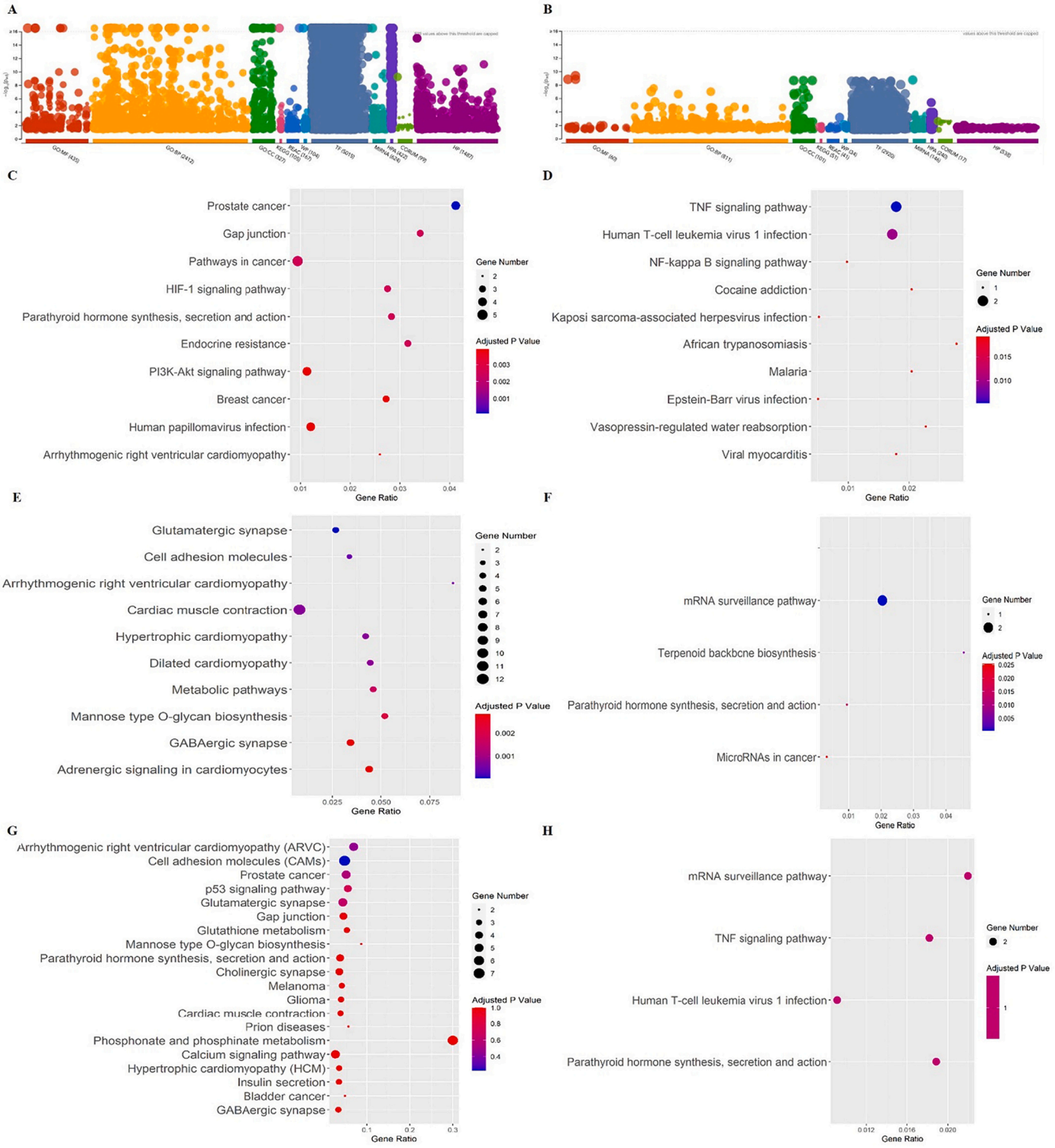

Fig. 4. KEGG Pathway analysis for coding DEGs in SH-MINCR+ and SH-siMINCR compared to SH-MOCK. In dot plot the y-axis represents the name of the pathway, the x-axis represents the Rich factor, dot size represents the number of different genes and the color indicates the adjusted p-value. G:Profiler plots for SH-MINCR+ (A) and SH-siMINCR (B). Dot plot of top 10 upregulated pathways in SH-MINCR+ (C) and SH-siMINCR (D) from g:Profiler analysis. Dot plot of top 10 downregulated pathways in SH-MINCR+ (E) and SH-siMINCR (F) from g:Profiler analysis. Dot plot of top 20 deregulated pathways in SH-MINCR+ from EnrichR analysis (G). Dot plot of deregulated pathways with at least 2 involved genes in SH-siMINCR from EnrichR analysis (H).

top 20 deregulated pathways are involved in inflammatory mechanisms (Supplementary Table 7). A full list of the 53 deregulated KEGG 2019 and of the 27 deregulated WikiPathways 2019 analysis for SH-siMINCR is reported in Supplementary Table 7.

2.2. *MINCR expression levels impact on oncogenesis and neurodegeneration*

2.2.1. *MINCR transcript levels and oncogenesis*

Since it has been demonstrated that MINCR acts in oncogenic

pathways in different types of cancer, we evaluated its effect on the modulation of the tumorigenic genes and pathways in SHSY-5Y neuroblastoma cell line. Using "The Human Protein Atlas (Pathology) – Scored Gene-Cancer Correlation" network of NDEx app [38,48], we were able to correlate deregulated coding genes and tumor prognosis. As shown in Fig. 5A-5B, 103 (51.76%) coding genes emerged as cancer related in SH-MINCR+ and 12 (46.5%) coding genes in SH-siMINCR.

In SH-MINCR+ the most deregulated cancer-related genes are involved in cell-signaling, cell-cell contact and in oxidative stress metabolism. PTPRK and DSG2 are the most upregulated genes. PTPRK is a tyrosine phosphatase enzyme involved in a variety of cellular processes including cell growth, mitotic cycle and tumorigenic transformation [23]. DSG2 encodes for the desmoglein-2 protein which is localized to desmosomes and functions to adhere adjacent cells together [2]. Except for DSG2 in colorectal cancer, where its presence influences positively the cancer status, the network shows that the upregulation of these genes leads to worse prognosis in pancreatic cancer (PTPRK) and in head and neck cancer and cervical cancer (DSG2), thus confirming an oncogenic role for MINCR overexpression. Moreover, the network reports the presence of favourable and unfavorable prognosis with a certain cancer type. Interestingly, genes with a favourable prognosis are strongly downregulated in SH-MINCR+, such as SHANK2 in renal cancer and PARVA in endometrial cancer, indicating its implication in the malignant tumor phenotype. SHANK2 is involved in postsynaptic density organization in excitatory neurons, but it is present also in other tissues where it localizes with the cyclic nucleotide phosphodiesterase PDE4D precluding cAMP/PKA signals [30], interestingly also PDE4D is downregulated in SH-MINCR+. PARVA is an actin-binding protein associated with focal contacts and plays a role in cell adhesion, motility and survival [47]. It has also been associated with lung cancer as an unfavorable prognostic factor.

In SH-siMINCR there is less tendency to tumorigenicity compared to SH-MINCR+. Indeed, as it is shown by the network (Fig. 5B), between the most deregulated genes there are downregulated factors usually upregulated in cancer and vice versa. For example, CCDC88C, a negative regulator of the Wnt signaling pathway [22], is upregulated in response to MINCR silencing leading to better outcome in lung cancer and head and neck cancer, but it is also an unfavorable gene in renal cancer. The other most upregulated gene is RET, a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor (GDNF) family [25], which is an unfavorable gene in endometrial cancer. NUS1 encodes for a subunit of cis-prenyltransferase and it is essential for dolichol synthesis and protein glycosylation [17], in the network it is reported as a disadvantageous gene in cervical cancer, but it is downregulated in SH-siMINCR. Like NUS1, also NUCKS1, whose presence is negative in endometrial cancer due to its action on DNA repair mechanism [21], is downregulated after MINCR silencing depicting an opposite scenario compared to tumor.

2.2.2. *MINCR transcript levels and neurodegeneration*

Since it has been demonstrated that numerous lncRNAs act in more than one type of pathology implementing different mechanisms, we wanted to elucidate MINCR possible pathways in other diseases. Given that the only available data about MINCR aside from cancer concerns neurodegenerative disorders [14] [61], we performed DEGs analysis through DisGeNET platforms [37]. As shown in Fig. 6A–6B, 48 (24.1%) coding genes emerged as involved in neurodegenerative diseases in SH-MINCR+ and 5 (19.2%) in SH-siMINCR.

In SH-MINCR+ (Fig. 6A) the most deregulated genes, ST8SIA1 and DSG2, are related to AD. ST8SIA1 is upregulated in response to MINCR overexpression and encodes for GD3 synthase which is responsible for biosynthesis of the b- and c-series gangliosides. In AD mouse model, the lack of GD3 inhibits Aβ-induced cell death and Aβ aggregation [4]. DSG2 rs8093731 variant has been classified as a top 20 risk variant in AD with brain amyloidosis and it seems to have an association with amyloid deposition [1]. Also, the two most downregulated genes in SH-MINCR+ are associated with AD, but, on the contrary, they have been found to be upregulated in the disease. SHANK2, a scaffold protein of glutamate excitatory synapses, is present at high levels leading to disruption of glutamate receptors in AD [16]. The second gene is KCNA3, a voltage-

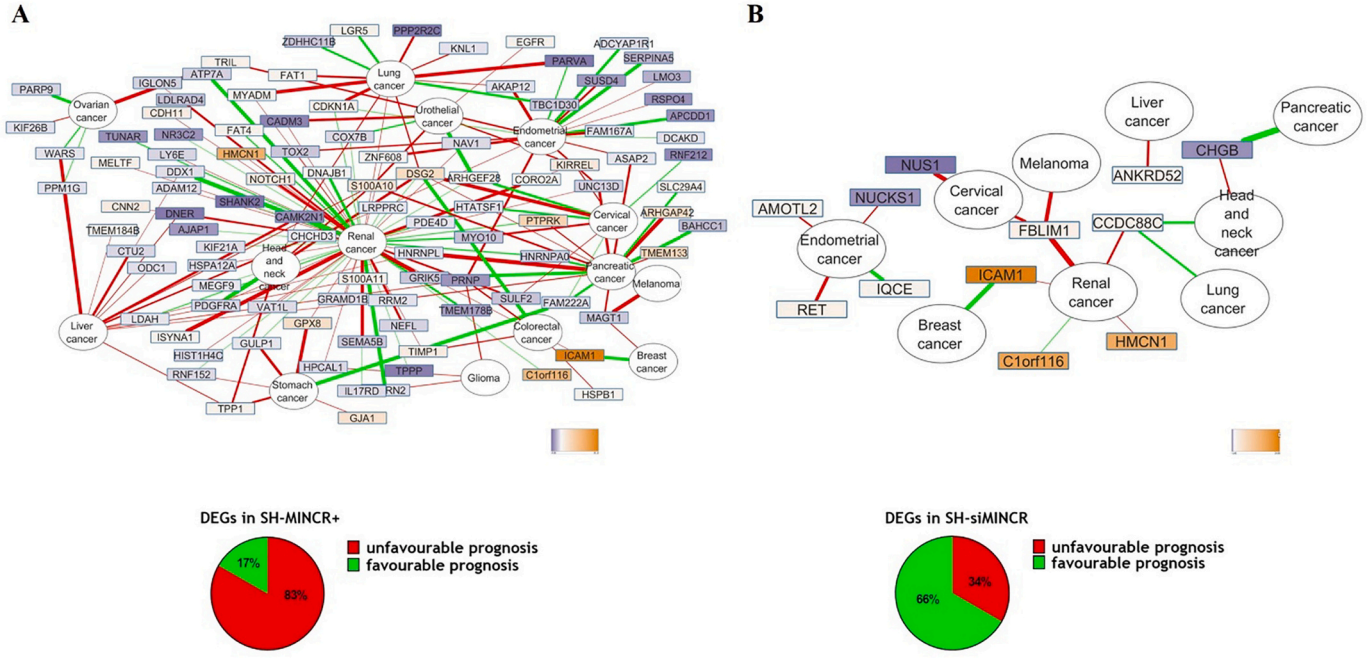

Fig. 5. MINCR-cancer correlations. DEGs associated with cancer in (A) SH-MINCR+ and (B) SH-siMINCR, performed through "The Human Protein Atlas (Pathology) – Scored Gene-Cancer Correlation" network, are shown. The network shows the correlation between mRNA expression level and patient's survival, the edge color represents prognostic information (green = favourable, red = unfavorable) while edge weight the correlation score, the gene color represents the Fold Change. Pie charts show the correlation between cancer and the 2 most up- and down-regulated genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

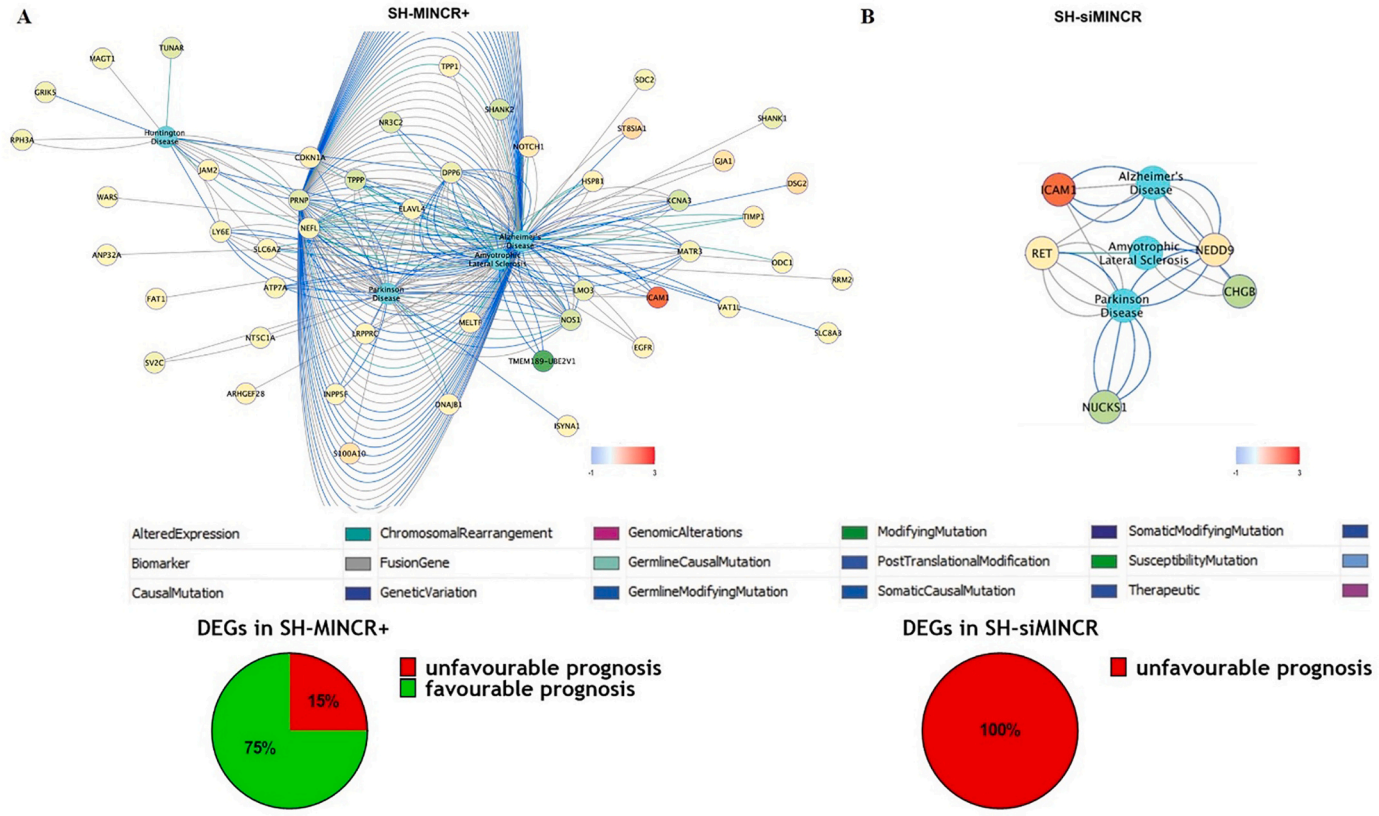

Fig. 6. MINCR-neurodegeneration correlations. DEGs associated with neurodegenerative diseases in (A) SH-MINCR+ and (B) SH-siMINCR performed through DisGeNET database are shown, the gene color represents Fold Change. Pie charts show the correlation between neurodegenerative diseases and the 2 most up- and down-regulated genes.

gated potassium channel, which is highly expressed on microglia during AD and promotes neuroinflammation, production of reactive oxygen species and amyloid-mediated microglial priming [40].

In SH-siMINCR (Fig. 6B) the most upregulated genes are RET and NEDD9. RET has been implicated in ALS where its increase in microglial cells in spinal cord of G93A mouse has been described, whilst its inhibition in human spinal cord motor neurons has been proposed as compensatory mechanism for preventing motor neuron degeneration [42]. Moreover, high levels of RET in microglia has been observed also in Parkinson's disease (PD) [52]. NEDD9 is involved in the outgrowth of neurites and polymorphisms and variations of this gene have been associated with AD and PD [31]. Upregulation of NEDD9 has also been described in monocytes of patients affected by rapidly progressive ALS [58]. The downregulated genes associated with neurodegenerative diseases are CHGB and NUCKS1. CHGB encodes a tyrosine-sulfated secretory protein, member of the granin family, whose downregulation in motor cortex of ALS patients leads to a reduction of glycolytic energy supply that constitutes one of the major causes for neuronal cell death [29]. Finally, after MINCR downregulation also NUCKS1 is downregulated and its decrease has been described in Parkinson's disease [45]. Taken together these data suggest that the downregulation of MINCR could be related to a neurodegenerative phenotype when compared to MINCR upregulation.

2.2.3. *MINCR up- and downregulation in the human nervous system*

To support our hypothesis that MINCR upregulation is related to oncogenesis while MINCR downregulation is implicated in neurodegenerative pathways, we investigated the expression levels of MINCR directly in the nervous system comparing biopsies of gliomas, spinal cord from sporadic ALS patients and hippocampal region from AD patients. Consistent with bioinformatic data, we found a significative upregulation of MINCR in glioma patients and, in contrast, MINCR downregulation in ALS (Fig. 7A–7B). Moreover, there is also a trend of down-regulation of MINCR expression in AD tissues, which supports data obtained from ALS tissue (Fig. 7C).

2.2.4. *MINCR deregulation impacts on cell proliferation, colony formation capacity, apoptosis and cytoskeleton abnormalities*

To support bioinformatic analyses, we performed functional in vitro studies demonstrating that cells overexpressing MINCR proliferate significantly more compared to cells transfected with empty vector (Fig. 8A), and that there is a visible increase in colony formation capacity of SH-MINCR+ (Fig. 8B), showing cancer-related features.

On the other hand, MINCR silencing reduced cells viability (Fig. 8C). Interestingly, in SH-siMINCR RNA-seq data we found upregulated PACS1, which promotes mitochondrial cell death [6], validated by qPCR (Fig. 8C). Moreover, we confirmed the cytoskeleton involvement found in GO analysis through qPCR evaluation of Nestin, which is an important neuronal cytoskeletal component (Fig. 8D).

3. Discussion

The deepening of our knowledge on the whole transcriptome is unveiling thousands of non-coding RNAs, which are showing a capacity to act in more than one fundamental cellular mechanism and interact with multiple molecules, depending on their expression levels. Thus, it is not surprising that their alterations lead to several diseases and, moreover, that the same lncRNA can cause different disorders, even almost biologically opposite to each other like cancer and neurodegenerative diseases. In this work we focused on the potential mechanisms exerted by MINCR expression levels in SH-SY5Y cells, an in vitro model presenting a neuronal phenotype. Indeed, we know that MINCR is generally

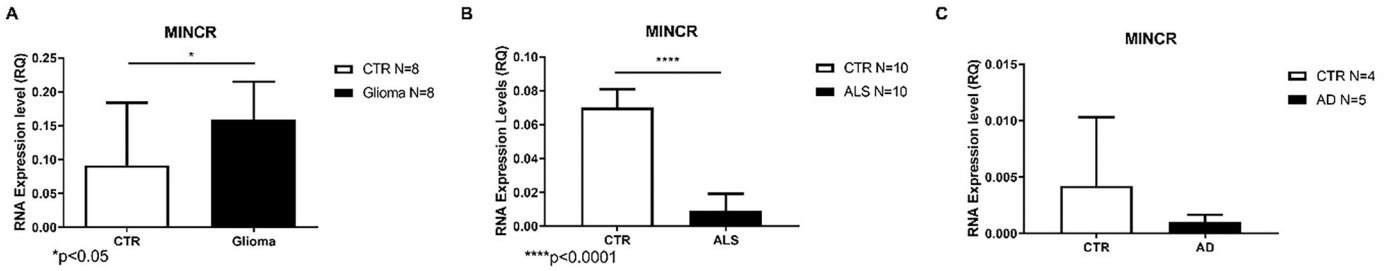

Fig. 7. MINCR expression levels in human central nervous system derived biopsies. (A) Upregulation of MINCR in brain biopsies of glioma affected patients compared to healthy control subjects. (B) Downregulation of MINCR in spinal cord tissue of sporadic ALS affected patients and (C) in hippocampus of AD affected patients compared to control healthy subjects.

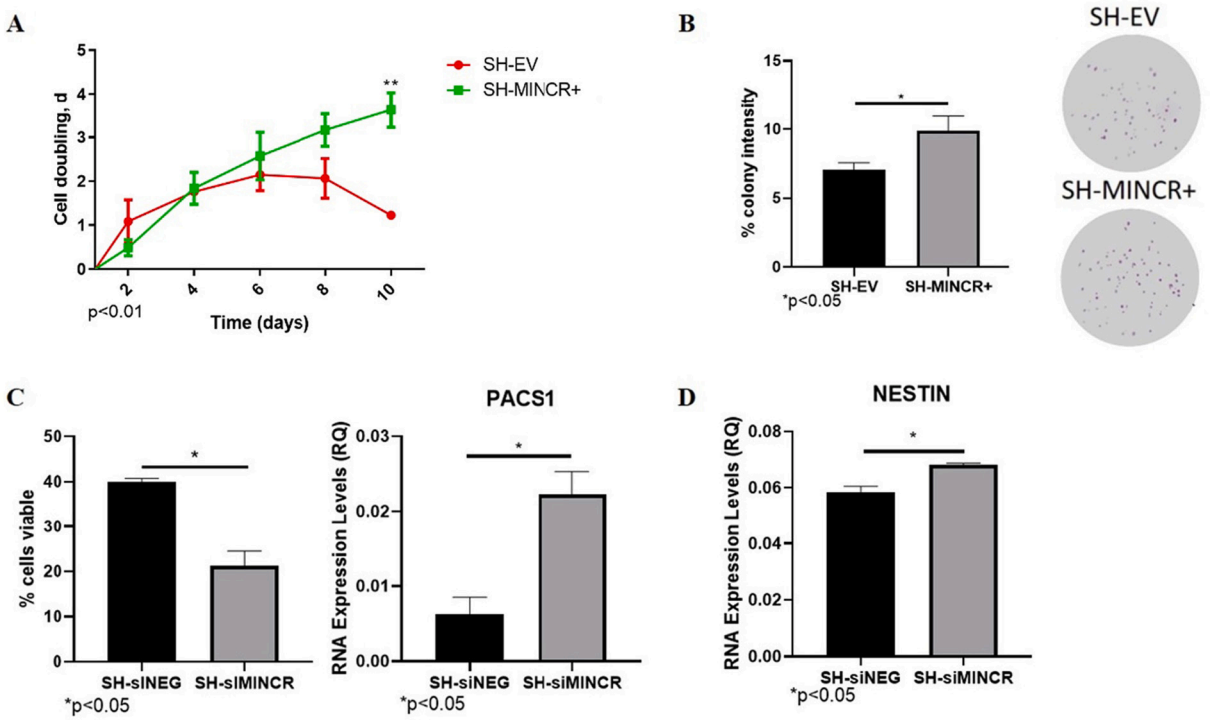

Fig. 8. In vitro studies recapitulate RNA-Seq findings. (A) MINCR overexpression increases cells proliferation ($n = 4$) and colony formation capacity ($n = 3$) (B) (SH-EV: SH-SY5Y transfected with empty vector). (C) MINCR downregulation reduces cell viability and (D) leads to cytoskeletal components alteration (SH-siNEG = 4 and SH-siMINCR = 4; SH-siNEG: SH-SY5Y transfected with negative control siRNA).

upregulated in cancer [54] and that it has been described as downregulated in ALS [14]. In addition to this, little is known about its mechanisms of action in cancer and almost no information is available on MINCR role in neurodegeneration.

RNA-sequencing analysis of SH-SY5Y cells overexpressing and silenced for MINCR allowed us to identify differentially deregulated RNAs. In general, MINCR overexpression caused an upregulation of cancer genes and a downregulation of genes implicated in functions important for the central nervous system. On the other hand, downregulation of MINCR led to an upregulation of genes implicated in RNA metabolism, a fundamental cellular process involved in neurodegenerative disease pathogenesis.

RNA sequencing pointed out a larger RNA perturbation in response to MINCR upregulation (227 DEGs) compared to MINCR downregulation (29 DEGs). Since the plasmid overexpression and the siRNA-mediated silencing both changed MINCR expression levels with a 3× factor, these data suggest a major role in multiple disruption for the overexpression of MINCR involving many different genes and pathways, while it seems that MINCR downregulation leads to smaller but more specific alterations. The presence of shared genes between SH-MINCR+ and SH-siMINCR, especially genes with many functions and thus involved in many different pathways, may suggest that MINCR dysregulation acts on a wide range of targets pursuing different undirect mechanisms of regulation that must be still clarified.

Through the analysis of the subcellular localization of DEGs derived proteins, we found that changes in MINCR expression levels cause alterations mainly in nuclear compartments in both cellular models. The localization of lncRNAs could be informative about the role and the types of mechanism carried out by an RNA transcript. As a predominantly nuclear lncRNA, MINCR could contribute to several biological processes, including regulation of gene expression and nuclear organization [46]. Intriguingly, some of the nuclear DEGs in SH-MINCR+ and SH-siMINCR are DNA binding proteins, such as zinc finger protein family members [9] (ZNF536, ZNF662, ZNF521, ZNF536 and ZNF608) deregulated in SH-MINCR+, NUCKS1 [21] in SH-siMINCR and the CAMP Responsive Element Binding Protein 5 (CREB5) [36] in both cell models.

Through g:Profiler, EnrichR and GO enrichment analyses, we were able to obtain a detailed profile of the pathways altered by MINCR expression level. The different outcomes caused by the upregulation and

downregulation of MINCR suggest that the lncRNA expression level can be a regulator of its activity. Indeed, according to literature, processes involved in cancer were the main enriched pathways in response to MINCR overexpression, specifically related to growth factor signaling (EGFR and PDGFR) and cell cycle (CDKN1A). Interestingly, CDKN1A is upregulated in SH-MINCR+ and it is an unfavorable factor in the types of cancers that emerged from pathway analysis: in prostate cancer CDKN1A overexpression is associated to worst clinical outcome [3], in gliomas CDKN1A expression is an indicator of shortened disease-free survival [27] and in bladder cancer CDKN1A is associated to invasiveness [26]. On the other hand, in response to MINCR downregulation, RNA metabolism and inflammation were the main altered pathways, highlighting two new MINCR related processes. In GO analysis the downregulation of MINCR also leads to the dysregulation of cytoskeleton, a critical structure for neurons. From that GO enriched terms, NEDD9 emerged as a deregulated gene involved in microtubule network stabilization and it is worthy to note that NEDD9 interacts for this function with Aurora A Kinase (AURKA) [26], which has been already demonstrated to be deregulated by MINCR silencing in BL-2 cells [13].

In support of the emerging mechanisms of MINCR overexpression and silencing, we analyzed DEGs involvement in cancer and neurodegenerative disorders. Even if SH-MINCR+ present DEGs that participate in neurodegeneration and SH-siMINCR DEGs are also involved in cancer, a clear trend emerged from the analysis showing a great role for MINCR overexpression in cancer and for MINCR downregulation in neurodegeneration. Some DEGs contribute to both pathological conditions in different ways according to their expression levels. For example, in SH-siMINCR we found PACS1 upregulated, a gene that promotes mitochondrial cell death regulating BAX/BAK oligomerization [6]. Mutations in PACS1 have been described in ALS patients [50]. Moreover, PACS1 participates also in tumor biology where, on the contrary, its downregulation promotes gastric cancer [7]. Another example is WDR82 that we found downregulated in SH-siMINCR. WDR82 is a component of the SET1A/SET1B histone H3-Lys4 methyltransferase complex and it has been found downregulated in ALS Drosophila model [5]. WDR82 is also upregulated in murine squamous cell carcinoma where it promotes the activity of pro-inflammatory genes [43]. Finally, the ambivalent behavior of this lncRNA in cancer and nervous system disorders has been proven both through MINCR expression levels in tissue derived from glioma, ALS and AD patients and through functional studies showing that MINCR overexpression leads to cancer-related features, while MINCR downregulation to apoptosis and cytoskeleton abnormalities, neurodegeneration-typical characteristics. This data supports the idea that cancer and neurodegeneration can share key genes and pathways conversely regulated and can be considered two sides of the same coin.

The findings here reported show that MINCR has more than one mechanism of action depending on its expression levels and suggest that it can participate in the pathogenesis of different diseases, not limiting its functions exclusively to cancer. The ability of a lncRNA to regulate different pathways only in relation to its abundance levels within the cells could be a new important perspective to explain the complexity of the lncRNA system. In MINCR case, this paradigm could even lead to really different pathologies. Therefore, understanding the cause and functional consequences of MINCR deregulation through a full characterization can give important information about pathogenetic mechanisms both in cancer and in neurodegeneration, suggesting that tools able to control this aspect could be a useful therapeutic strategy. Although this study is preliminary in the sense that it highlights potential target of MINCR action, further experiments will allow the full characterization of MINCR mechanism and implications in both cancer and neurodegeneration biology, potentially becoming a novel targetable gene in the diseases.

4. Materials and methods

4.1. Cell culture and transfection

The human neuroblastoma cell line SH-SY5Y (ATCC) was grown in DMEM (Sigma–Aldrich) supplemented with 15% fetal bovine serum (Carlo Erba), 1% L-Glutammine (Carlo Erba) and 1% penicillin/streptomycin (Carlo Erba), at 37 °C in a 5% CO2 atmosphere. The formal certification of STR profiling for SH-SY5Y cell line from ATCC is the following:

Amelogenin: X
CSF1PO: 11
D13S317: 11
D16S539: 8,13
D5S818: 12
D7S820: 7,10
THO1: 7,10
TPOX: 8,11
vWA: 14,18

For MINCR stable transfection, a pCMV6-AC-GFP construct with or without MINCR sequence (OriGene) was used. We cloned MINCR longest isoform (Refseq NR_120682.1). The plasmid was transformed in *E. Coli*, individual colonies were checked for successful ligations by growing cultures, DNA purification, agarose gel run and sequence. For transfection, cells were seeded in 24-well plates at a density of 6×10^4 cells/well. After 24 h, cells were transfected with 500 ng of plasmid and FugeneHD transfection reagent (Roche) following manufacturer's instructions. After 48 h incubation, the culture medium was changed, and cells were cultured with a selective medium containing 600 μg/ml gentamicin-disulfate G418 (Roth). After 3 weeks of G418 selection, single clones were isolated and cultured in 48-well plate. After screening of the clones, we chose four independent clones that overexpressed MINCR at similar levels to further analysis. The transfection efficiency was observed through qPCR (Supplementary fig. 1) and calculating the % of GFP positive cells observed by fluorescence microscopy through ImageJ "Cell counter" plug-in as $\%Transfection\ efficiency = \frac{number\ of\ GFP\ positive\ cells}{total\ number\ of\ cells\ per\ field}\ x\ 100$ analyzing 3 different fields for each of the 4 clones (Supplementary fig. 2).

For MINCR silencing, SH-SY5Y cells were seeded in a 6 well-plate at a density of 4×10^5 cells/well the day before transfection. The cells were transiently transfected with 5 nM siRNA pre-designed by the company on MINCR longest isoform (ThermoFisher) using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. We used two different siRNAs: one for RNA-seq analysis and one for validation and functional experiments. We tested 3 different time points to achieve the best transfection efficiency (Supplementary Fig. 2) and we decided to harvest the cells after 72 h of transfection.

4.2. RNA extraction

Total RNA from SH-MOCK, SH-MINCR+, SH-siMINCR, human brain and spinal cord tissue was isolated using Trizol® reagent (Life Science Technologies) following manufacturer's specifications. RNAs were quantified and examined using Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies) and 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).

Cytoplasmic and nuclear RNA fractions were extracted using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek Corp.).

4.3. cDNA synthesis and qPCR

Total cDNAs were prepared from 1 μg of total, nuclear and cytoplasmic RNA using iScript™ cDNA Synthesis Kit (Bio-Rad). qPCR was performed according to manufacturer's instructions with iQ™ SYBR®

Green Supermix (Bio-Rad). Primers were designed with Primer3Plus software [49]. List of primers is available in Supplementary Table 8 and qPCR validation in Supplementary Fig. 3. Genes were quantified in triplicates, GAPDH was used as housekeeping gene. Gene expression was calculated using the 2-ΔCt method.

4.4. Library preparation for RNA-seq and bioinformatic data analysis

Libraries from SH-MOCK, SH-MINCR+ and SH-siMINCR were prepared with the SENSE Total RNA-Seq Library Prep Kit (Lexogen) using 500 ng total RNA and sequenced by Illumina NextSeq 550 sequencer. The quality of sequencing libraries was assessed by 2100 Bioanalyzer with DNA1000 assay (Agilent) and quantified with Qubit dsDNA HS Assay Kit (Invitrogen). FastQ files were generated via Illumina bcl2fastq2 starting from raw sequencing reads produced by Illumina NextSeq sequencer (Version 2.17.1.14- http://support.illumina.com/downloads/bcl-2fastq-conversion-software-v217.html). Gene and transcript intensities were computed using STAR/RSEM software using Gencode Release 27 (GRCh38) as a reference, using the "stranded" option. Differential expression analysis for mRNA was performed using R package DESeq.2. Coding and non-coding genes were considered differentially expressed and retained for further analysis with $|\log2(\text{SH-MINCR+/SH-MOCK})| \geq 1$ and a False Discovery Rate (FDR) ≤ 0.1.

4.5. Pathway analysis and gene ontology

We performed KEGG pathway analysis (Kyoto Encyclopedia of Genes and Genomes http://www.genome.ad.jp/KEGG), WikiPathways analysis and Gene Ontologies of differentially expressed coding genes via EnrichR web tool [28] and g:Profiler [41], ranking terms according to their fold change and using a Bonferroni-Hochberg FDR of 0.05 as threshold. The R software was used to generate Dotplot graphs (with the ggplot2 library) and GOChord graphs (with the GOplot library). All other representations of functional enrichment were generated using the Cytoscape software [44] and the following plugins included in NDEx [38]: "The Human Protein Atlas (Cell) - Protein Sub-cellular Localization" network [48],"The Human Protein Atlas (Pathology) – Scored Gene-Cancer Correlation" network [48] and DisGeNET [37]. We used default settings with minor changes. Briefly, we excluded "uncertain" gene from analysis with "The Human Protein Atlas (Cell) - Protein Subcellular Localization" network and we used the "CURATED" database for DisGeNET analysis.

4.6. Patients and control subjects

Brain tissue samples were obtained from 8 glioma patients and 8 age- and sex-matched controls (Protocol n° 20,180,008,163 version 04/01/2018). Spinal cord tissue samples from 10 sporadic unmutated ALS patients and 10 healthy subjects were provided by the Human Brain and Spinal Fluid Resource Center (VA West Los Angeles Healthcare center, Los Angeles, CA 90073), which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society, and Department of Veteran Affairs. Hippocampus samples from AD patients and controls have been provided by Abbiategrasso Brain Bank, the study protocol was approved by the Ethics Committee of the University of Pavia on October 6th, 2009 (Committee report 3/2009).

4.7. Cell viability, cell proliferation and colony formation assay

Cell viability was assessed using trypan blue exclusion test. Briefly, cells resuspended in trypan blue were transferred in a Cell Counting Slides (Bio-Rad) and scored as able (live) or unable to exclude the dye (dead) through TC20™ Cell Counter (Bio-Rad).

For cell proliferation, transfected cells were plated in 24-well at a density of 5×10^4 cells per well. Cells were counted every 2 days for 10 days in 4 experiments ($N = 4$) through trypan blue exclusion method, as previously described. To evaluate the growth, the cell doubling time was calculated with the following formula:

$$d = \frac{ln\frac{X_f}{X_i}}{ln2}$$

where X_f and X_i were the final and the initial number of cells for each condition, respectively.

For colony formation assay, transfected cells (0.5×10^3 cells per well) were seeded in a six-well plate. After 15 days, colonies were fixed with 4% paraformaldehyde for 20 min and subsequently washed with PBS for 3 times. Then stained for 30 min with 0.5% crystal violet (Sigma-Aldrich) and the number and intensity of colonies was obtained with the "ColonyArea" ImageJ plug-in [20].

4.8. Statistical analysis

Two-tailed student's *t*-test was performed for statistical analysis. The Prism 8 software (GraphPad Software Inc.) was used assuming a *P* value less than 0.05 as the limit of significance.

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Author statement

C.P. wrote the manuscript. C.P. and M.G. performed the experiments. C.P., M.G. and F.R. analyzed the data. S.Z. performed the bioinformatic analysis. G.B., A.D. and T.E.P. participated to patients and controls recruitment. J.G., D.S., M.B., O.P. and S.C. participated to experimental plan and data discussion. S.G. and C.C. supervised this work and reviewed the manuscripts. All authors reviewed and accepted the final version of this manuscript.

Author contributions

C.P. wrote the manuscript. C.P. and M.G. performed the experiments. C.P., M.G. and F.R. analyzed the data. S.Z. performed the bioinformatic analysis. G.B., A.D. and T.E.P. participated to patients and controls recruitment. J.G., D.S., M.B., O.P. and S.C. participated to experimental plan and data discussion. S.G. and C.C. supervised this work and reviewed the manuscripts. All authors reviewed and accepted the final version of this manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest. S.Z. has shares of enGenome, an italian bioinformatics company. The funders and enGenome had no role in the design of the study, in the collection, analyses or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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