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ANÁLISIS COMPARATIVO DE LA EXPRESIÓN DE miRNAs EN EL DESARROLLO EMBRIONARIO DEL COLON, EL CÁNCER COLORECTAL Y EL LINFOMA DE HODGKIN

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ANEXO 1

Tablas suplementarias

Tabla suplementaria 1. Secuencias de los miRNAs analizados en esta tesis.		
miRNA	*Secuencia del miRNA maduro*	*Referencias*
hsa-miR-9	ucuuugguuaucuagcuguauga	
hsa-miR-9*	uaaagcuagauaaccgaaagu	
hsa-miR-10a	uacccuguagauccgaauuugug	
hsa-miR-15a	uagcagcacauaaugguuugug	Calin et al. 2005. N. Engl. J. Med
hsa-miR-15b	uagcagcacaucaugguuuaca	
hsa-miR-17-3p	acugcagugaaggcacuugu	Hayashita et al. 2005. Cancer Res. Yanaihara et al. 2006. Cancer Cell*
hsa-miR-17-5p	caaagugcuuacagugcagguagu	Hayashita et al. 2005. Cancer Res. Volinia et al. 2006. Proc. Natl. Acad. Sci *
hsa-miR-19a	ugugcaaaucuaugcaaaacuga	Hayashita et al. 2005. Cancer Res
hsa-miR-20	uaaagugcuuauagugcaggua	Hayashita et al. 2005. Cancer Res. Garzon et al. 2006. Proc. Natl. Acad. Sci.
hsa-miR-21	uagcuuaucagacugauguuga	Volinia et al. 2006. Proc. Natl. Acad. Sci *. Yanaihara et al. 2006. Cancer Cell*
hsa-miR-23a	aucacauugccagggauuucc	
hsa-miR-23b	aucacauugccagggauuaccac	Calin et al. 2005. N. Engl. J. Med
hsa-miR-25	cauugcacuugucucggucuga	Volinia et al. 2006. Proc. Natl. Acad. Sci *
hsa-miR-26a	uucaaguaauccaggauaggcu	
hsa-miR-26b	uucaaguaauucaggauaggu	
hsa-miR-27a	uucacaguggcuaaguuccgcc	
hsa-miR-27b	uucacaguggcuaaguucug	
hsa-miR-28	aaggagcucacagucuauugag	
hsa-miR-29a	cuagcaccaucugaaaucgguu	Calin et al. 2005. N. Engl. J. Med. Pekarsky et al. 2006. Cancer Res.
hsa-miR-29b	uagcaccauuugaaaucagu	Calin et al. 2005. N. Engl. J. Med. Volinia. Pekarsky et al. 2006. Cancer Res. et al. 2006. Proc. Natl. Acad. Sci

hsa-miR-128a	ucacagugaaccggucucuuuu	Volinia et al. 2006. Proc. Natl. Acad. Sci *
hsa-miR-128b	ucacagugaaccggucucuuuc	
hsa-miR-129	cuuuuugcggucugggcuugc	
hsa-miR-130a	cagugcaauguuaaaagggc	
hsa-miR-130b	cagugcaaugaugaaagggcau	
hsa-miR-132	uaacagucuacagccauggucg	
hsa-miR-133a	uuggucccuucaaccagcugu	
hsa-miR-133b	uuggucccuucaaccagcua	
hsa-miR-134	ugugacugguugaccagaggg	
hsa-miR-135a	uauggcuuuuuauuccuauguga	
hsa-miR-135b	uauggcuuuucauuccuaugug	
hsa-miR-137	uauugcuuaagaauacgcguag	
hsa-miR-138	agcugguguugugaauc	
hsa-miR-139	ucuacagugcacgugucu	
hsa-miR-140	agugguuuuacccuaugguag	
hsa-miR-141	aacacugucugguaaagaugg	
hsa-miR-142-3p	uguaguguuuccuacuuuaugga	
hsa-miR-142-5p	cauaaaguagaaagcacuac	
hsa-miR-144	uacaguauagaugauguacuag	
hsa-miR-145	guccaguuuucccaggaaucccuu	Yanaihara et al. 2006. Cancer Cell*
hsa-miR-146	ugagaacugaauuccauggguu	Calin et al. 2005. N. Engl. J. Med. Volinia et al. 2006. Proc. Natl. Acad. Sci *
hsa-miR-147	guguguggaaaugcuucugc	
hsa-miR-148a	ucagugcacuacagaacuuugu	
hsa-miR-149	ucuggcuccgugucuucacucc	
hsa-miR-150	ucucccaacccuuguaccagug	
hsa-miR-151	acuagacugaagcuccuugagg	
hsa-miR-152	ucagugcaugacagaacuugg	
hsa-miR-154	uagguuauccguguugccuucg	
hsa-miR-154*	aaucauacacgguugaccuauu	

hsa-miR-205	uccuucauuccaccggagucug	
hsa-miR-210	cugugcgugugacagcggcug	
hsa-miR-211	uucccuuugucauccuucgccu	
hsa-miR-213	accaucgaccguugauuguacc	
hsa-miR-214	acagcaggcacagacaggcag	Volinia et al. 2006. Proc. Natl. Acad. Sci *
hsa-miR-215	augaccuaugaauugacagac	
hsa-miR-216	uaaucucagcuggcaacugug	
hsa-miR-218	uugugcuugaucuaaccaugu	
hsa-miR-219	ugauuguccaaacgcaauucu	
hsa-miR-220	ccacaccguaucugacacuuu	
hsa-miR-221	agcuacauugucugcuggguuuc	Calin et al. 2005. N. Engl. J. Med. Volinia et al. 2006. Proc. Natl. Acad. Sci *. Felli et al. 2005. Proc. Natl. Acad. Sci.
hsa-miR-222	agcuacaucuggcuacugggucuc	Felli et al. 2005. Proc. Natl. Acad. Sci.
hsa-miR-223	ugucaguuugucaaauacccc	Calin et al. 2005. N. Engl. J. Med. Volinia et al. 2006. Proc. Natl. Acad. Sci *. Fazi et al. 2005. Cell.
hsa-miR-224	caagucacuagugguuccguuua	
hsa-miR-296	agggccccccucaauccugu	
hsa-miR-299	ugguuuaccgucccacauacau	
hsa-miR-301	cagugcaauaguauugucaaagc	
hsa-miR-302a	uaagugcuuccauguuuugguga	
hsa-miR-302b	uaagugcuuccauguuuuaguag	
hsa-miR-302b*	acuuuaacauggaagugcuuucu	
hsa-miR-302c	uaagugcuuccauguuucagugg	
hsa-miR-302c*	uuuaacauggggguaccugcug	
hsa-miR-302d	uaagugcuuccauguuugagugu	
hsa-miR-320	aaaagcuggguugagagggcgaa	
hsa-miR-323	gcacauuacacggucgaccucu	
hsa-miR-324-5p	cgcaucccuagggcauuggugu	
hsa-miR-325	ccuaguagguguccaguaagu	

hsa-miR-326	ccucugggcccuuccuccag	
hsa-miR-328	cuggcccucucugcccuuccgu	
hsa-miR-330	gcaaagcacacggccugcagaga	
hsa-miR-331	gccccugggccuauccuagaa	
hsa-miR-335	ucaagagcaauaacgaaaaaugu	
hsa-miR-337	uccagcuccuauaugaugccuuu	
hsa-miR-338	uccagcaucagugauuuuguuga	
hsa-miR-339	ucccuguccuccaggagcuca	
hsa-miR-340	uccgucucaguuacuuuauagcc	
hsa-miR-342	ucucacacagaaaucgcacccguc	
hsa-miR-367	aauugcacuuuagcaaugguga	
hsa-miR-368	acauagaggaaauuccacguuu	
hsa-miR-370	gccugcuggguggaaccugg	
hsa-miR-371	gugccgccaucuuuugagugu	
hsa-miR-372	aaagugcugcgacauuugagcgu	
hsa-miR-373	gaagugcuucgauuuuggggugu	
hsa-miR-373*	acucaaaaugggggcgcuuucc	
hsa-miR-374	uuauaauacaaccugauaagug	
hsa-let-7a	ugagguaguagguuguauaguu	Yanaihara et al. 2006. Cancer Cell*
hsa-let-7b	ugagguaguagguuguguggu	Yanaihara et al. 2006. Cancer Cell*
hsa-let-7d	agagguaguagguugcauagu	
hsa-let-7e	ugagguaggagguuguauagu	
hsa-let-7g	ugagguaguaguuuguacagu	
hsa-let-7i	ugagguaguaguuugugcu	
hsa-miR-16	uagcagcacguaaauauuggcg	
cel-miR-2	uaucacagccagcuuugaugugc	
cel-lin-4	ucccugagaccucaaguguga	
ath-miR159a	uuuggauugaagggagcucua	

*Tumor sólido

Tabla Suplementaria 2. Expresión de los miRNAs en todas las muestras y fold change en tejido normal vs tumoral de colon y embrionario de 7-8 semanas vs 9-12 semanas. 13 miRNAs no se expresaron ninguna de las muestras: miR-104, miR-144, miR-220, miR-302a, miR-302b, miR-302b*, miR-302c*, miR-302d, miR-367, miR-371, miR-372, miR-373*

	Media Tejido Normal	Media Tejido Tumoral	Fold Change (T/N)	Media Tejido Embrionario 7-8 semanas	Media Tejido Embrionario 9-12 semanas	Fold Change (7/12)
miR-9	-0.018573415	-0.459426	0.736699115	1.4745843	0.6296427	1.796192035
miR-9*	-0.377302	-0.7104885	0.793781311	0.6671659	0.02863597	1.556742067
miR-10a	0.068019465	0.3856405	1.246273782	1.4909203	0.05046884	2.714057835
miR-15a	-0.82733464	-0.34067628	1.401195597	0.20542741	-0.9024134	2.155228465
miR-15b	-0.8906063	-0.5320372	1.282153596	0.6559059	-0.46433297	2.173829621
miR-16	-1.0824391	-0.81796956	1.201194305	-0.23894918	-1.1940545	1.93872116
miR-17-3p	-0.7293203	-0.07361598	1.575384864	0.7043458	-0.46418127	2.247820868
miR-17-5p	-0.8402405	-0.07163682	1.703620128	1.3366475	-0.33059195	3.176062824
miR-20	-0.85608786	-0.15353155	1.627385803	0.9559054	-0.54173493	2.823804734
miR-21	-0.42399678	0.43028387	1.807857105	-0.38783872	-1.1164695	1.657065672
miR-23a	0.053584397	0.1350683	1.05810581	0.39737678	-0.40900436	1.748819194
miR-23b	0.47335958	0.39498916	0.947126859	1.6174109	0.7514971	1.822493665
miR-25	-0.5356308	-0.07065165	1.380297405	0.9201263	-0.234634	2.226473283
miR-26a	-0.12140764	-0.1636834	0.971121855	0.8669354	-0.04045322	1.87564737
miR-26b	-0.28660035	-0.13348539	1.11196776	0.527062	-0.27608797	1.744906787
miR-27a	0.037474543	0.43331745	1.315711251	0.30276728	-0.68217796	1.979238186
miR-27b	-0.20042245	0.011997867	1.158630314	0.47234938	-0.39621803	1.82584894
miR-28	0.007794737	0.17679526	1.124279331	0.6983817	-0.20331882	1.868266832
miR-29a	-0.35602108	0.2608046	1.533497357	-1.5104456	-2.0815759	1.485687098
miR-29b	-0.09074347	0.3018132	1.312717675	-0.9831791	-1.8200363	1.7861549
miR-29c	-0.026728846	0.2475768	1.209411872	-0.41741973	-1.2812632	1.819880183
miR-301	-0.57188296	-0.15699248	1.333197466	1.9939181	0.77600235	2.32610424
miR-30a-3p	0.2834813	-0.20884128	0.710879738	1.2388965	-0.03988582	2.426341001
miR-30b	0.15755096	0.10657155	0.965280801	1.2971942	0.2530078	2.062203062
miR-30c	0.1870861	0.06603149	0.919515239	1.4700893	0.350836	2.172345087
miR-30d	-0.3422279	-0.06897863	1.208526636	0.5354652	-0.34678105	1.843242964
miR-30e	-0.4251019	-0.3904166	1.024333357	0.022627287	-0.9064773	1.904093848
miR-31	-0.20333551	1.2854769	2.806578496	2.2521129	1.3190514	1.909323418
miR-34a	-0.23841283	0.34403527	1.497388002	-0.27954093	-1.299409	2.027733521
miR-34b	-0.27362442	-0.09754143	1.129812204	0.42300472	-0.56885386	1.988745381
miR-34c	-3.83E-04	0.6314818	1.549566908	0.78790003	-0.47477806	2.399407323
miR-92	-0.5060395	0.08876553	1.51026847	1.1622268	-0.150168	2.48353452
miR-95	-0.4497543	0.4429052	1.856595466	0.45576206	-0.49536535	1.933382931
miR-96	-1.1405791	0.16478123	2.471454472	0.2715792	-0.6291416	1.866998541
miR-98	-0.32655916	0.05776929	1.305252073	-0.92081875	-0.16763328	0.593292121
miR-99a	0.29793254	-0.49611497	0.57672381	0.8956976	0.43916583	1.372238996
miR-100	0.24406798	-0.25355354	0.708273505	1.1220423	0.5645977	1.471660202
miR-103	-0.36927435	-0.009443049	1.283275831	1.4050776	0.09097451	2.486477007
miR-105	-0.64439553	-0.0761292	1.482740707	1.3737897	0.11837986	2.387349587
miR-106a	-0.47357622	0.3885364	1.817698121	1.7413166	0.09619751	3.127736747
miR-107	-0.20511678	0.27441776	1.39429375	1.9018806	0.55593354	2.541970099

miR-122a	-0.118017115	0.36605552	1.3986865	1.9150648	1.0539864	1.816395541
miR-124a	-0.23596975	-0.62704355	0.762561817	1.2570353	0.7418608	1.429166993
miR-124b	-0.1008555	0.25031367	1.275593959	0.6382234	0.34727287	1.223446088
miR-125a	0.009014088	-0.19029325	0.87096863	1.4551765	0.35371944	2.145712909
miR-125b	0.43339166	-0.10026835	0.690800001	1.296648	0.72464293	1.486588209
miR-126	-0.0799142	-0.07633433	1.002484458	0.7821547	-0.365886	2.216127214
miR-127	-0.18772176	7.80E-04	1.13957985	1.6019516	0.8767887	1.653087282
miR-128a	-0.39501026	-0.06718197	1.255122597	1.0985551	0.02522006	2.10429218
miR-128b	-0.35239533	-0.32090396	1.022068131	1.0924673	0.17014462	1.895163975
miR-129	-3.70E-04	-0.35505503	0.782040346	0.6611534	0.0833656	1.492558833
miR-130a	0.15781233	-0.10990802	0.830631017	1.9539871	0.7136443	2.362546623
miR-130b	-0.29645756	0.2109075	1.421451682	2.0926442	0.6852694	2.652540539
miR-132	-0.06349586	0.19527383	1.196457947	0.8535396	-0.02568004	1.839380097
miR-133a	0.30341244	-0.593096	0.537185236	1.1571013	0.30527905	1.80477908
miR-133b	0.050610088	-0.94457024	0.501673164	0.9603137	0.14882332	1.755023538
miR-134	0.06745343	0.4567512	1.309755727	2.0975819	1.2388309	1.813467638
miR-135a	-0.33839464	0.6341462	1.962293498	2.5879579	1.4175811	2.250704727
miR-135b	-0.64290756	1.5521406	4.579049498	1.7366941	1.0105194	1.654247042
miR-137	-0.17998381	-1.0862179	0.533576085	1.45913	1.0703424	1.309292649
miR-138	-0.58155644	-0.42607158	1.113795879			
miR-139	0.47689217	-0.2160248	0.61860184	0.48274052	-0.42926154	1.881654902
miR-140	0.29165506	0.29491487	1.002262083	1.3804144	0.27577928	2.150444831
miR-141	-1.2923019	0.17359884	2.762358835	-0.3616073	-0.8679409	1.420435773
miR-142-3p	-0.90777	-0.37588063	1.445821421	-0.51419	-1.4981536	1.977891929
miR-142-5p	-0.65957993	-0.25206435	1.326399695	-0.500415	-1.8926526	2.624854766
miR-145	0.33997473	-0.48893616	0.562954064	0.51376784	-0.27872035	1.732059139
miR-146	-0.45878533	0.34540525	1.746165836	-0.5381804	-1.4375454	1.865244818
miR-147	-1.0120136	-0.4086641	1.519239689			
miR-148a	-0.62185746	0.27679163	1.864319455	0.38501605	-0.78290087	2.246870413
miR-149	0.057776242	-0.21929267	0.825265988	1.9329603	0.8970432	2.050416652
miR-150	-0.32486534	-0.26584142	1.041760701	-1.1336124	-1.9306098	1.737481239
miR-151	-0.14077961	0.25482887	1.315497476	1.2161956	0.08493332	2.190503136
miR-152	0.06734395	-0.050793737	0.921376248	0.5112433	-0.04054921	1.465905911
miR-154	-0.6478445	-0.58757174	1.042662871	0.89329624	0.31997982	1.48794007
miR-154*	-0.49110195	-0.05311655	1.354711268	1.7772996	1.0035731	1.709680211
miR-155	-0.66790295	-0.043268908	1.541819674	-0.0439346	-0.73851997	1.618419233
miR-181a	-0.5100289	-0.07120961	1.355494529	1.3768829	0.39766178	1.971400805
miR-181b	-0.6523042	-0.06965602	1.497595682	1.4532428	0.3489342	2.149958184
miR-181c	-0.57980967	-0.207699	1.294244933	0.94726574	0.1736017	1.709606194
miR-182	-1.4279151	-0.470481	1.941853146	-0.14175054	-0.9699023	1.775409424
miR-182*	-1.0135515	0.33374318	2.54434566			
miR-183	-1.1891028	0.20056728	2.620187547	0.6583036	-0.3477062	2.008348729
miR-184	-0.3340623	-0.5986684	0.832425982			
miR-185	-1.1219946	-0.89608335	1.169515717	-1.0981281	-1.8341204	1.665542652
miR-186	-0.67324305	-0.32027358	1.277186731	0.53715175	-0.58476335	2.176356805
miR-187	0.6919146	-0.19251534	0.541701526	1.9208814	0.5488885	2.588278571
miR-189	-0.24706891	-0.37234497	0.916828591	0.29406995	-0.50344306	1.738102315
miR-190	-0.23249102	0.16663036	1.318704558	1.517401	0.47802177	2.05534308
miR-191	-0.7656252	-0.22668174	1.452908108	0.21506688	-0.7800043	1.993178863
miR-193	-0.116117135	-0.29694188	0.882198526	-0.04399207	-1.0776006	2.047138239
miR-194	-1.5518861	-0.15258391	2.637739678	-0.97942406	-1.0940033	1.082659243
miR-195	-0.4158001	-0.7088521	0.816173628	-0.792525	-1.8075948	2.02100067

miR-197	-0.6876444	-0.42229992	1.201923005	0.5079414	-0.4669248	1.965458906
miR-198	-0.7305603	-0.42558503	1.23539746	-0.6168159	-0.8555724	1.179975169
miR-199a	-0.49089772	-0.66042376	0.889134735	0.9626914	0.12412345	1.788274176
miR-199a*	-0.58901376	-0.57649	1.008718596	0.60780674	-0.16532661	1.708977437
miR-199b	-0.51499796	-0.67225254	0.896729907	0.9364857	0.44470358	1.406180818
miR-199-s	-0.66885424	-0.5760991	1.066404771	0.51930285	-0.1871516	1.63178892
miR-19a	-0.9276126	-0.09190239	1.784735412	0.81267285	-0.49332485	2.47254658
miR-200a	-1.8683277	-0.24880454	3.072734594	-0.66049325	-1.161932	1.415624612
miR-200b	-1.7785051	-0.1410023	3.111268276	-0.5793253	-1.1342996	1.469142462
miR-200c	-1.7579842	-0.15472144	3.038296698	-0.35290486	-0.8320486	1.393916112
miR-203	-1.6626562	0.059655566	3.29964717	-0.9851613	-1.0010085	1.011044992
miR-204	-0.21468496	-0.42570862	0.863924019	0.7125083	0.28817996	1.341947601
miR-205	-1.0562638	-0.56457907	1.406085896	2.5995047	2.0919983	1.421590948
miR-210	-0.87513995	0.17660344	2.073033442	0.7056102	-0.10127887	1.749435009
miR-211	0.61618257	0.6951592	1.056268515			
miR-213	-0.3809197	0.16609019	1.461054394	1.184822	0.29285333	1.855706653
miR-214	-0.17979781	-0.2055066	0.982337862	1.4387829	0.4366108	2.003013438
miR-215	-1.0619845	0.17462376	2.356438877	-0.25768784	-0.38883677	1.095165519
miR-216	0.11331932	0.8091802	1.619850736			
miR-218	-0.2102134	-0.5962892	0.765208179	1.8246084	0.6844204	2.204097433
miR-219	-0.3841036	0.19245628	1.491289013	1.0104884	0.34701347	1.583893058
miR-221	-0.36793026	0.33505923	1.627874511	1.168766	0.1144791	2.076691479
miR-222	-0.44156748	0.22602661	1.588421826	0.732533	-0.25767288	1.98646845
miR-223	-0.7270241	-0.33464155	1.312559251	-0.6798982	-1.8297586	2.218924223
miR-224	-0.21909823	0.6674222	1.848711919	0.8111909	0.00740623	1.74567461
miR-296	-0.774619	-0.74481344	1.020874528	1.1538827	-0.0507969	2.304860757
miR-299	-0.48696396	-0.2671263	1.164602532	1.5376056	0.99968797	1.451875383
miR-320	-0.6547979	-0.30169892	1.277301389	0.5976692	-0.44333002	2.057652303
miR-323	-0.6576153	-0.53020227	1.092333226	1.7322764	0.74207157	1.986467004
miR-324-5p	-0.5103038	-0.13926789	1.293281122	1.0877038	0.08427444	2.004759757
miR-325	-0.6262544	-0.27005163	1.280052312			
miR-326	-0.32307652	-0.031556245	1.223929344	1.177145	0.04780431	2.187587454
miR-328	-0.23472811	-0.45882097	0.856133177	0.88461125	-0.17451979	2.08367611
miR-330	-0.40690526	-0.009642953	1.317006355	1.0895625	-0.09791673	2.27754448
miR-331	-0.20092297	7.46E-04	1.150028311	1.3724109	0.16698313	2.306056355
miR-335	-0.2779682	-0.07720679	1.149304763	1.5949287	0.27817225	2.491054266
miR-337	-0.72873485	-0.20609623	1.436580283	1.175861	0.4290848	1.678038946
miR-338	-0.7855991	-0.40727097	1.299834666	0.1371081	-0.5166018	1.573208515
miR-339	-0.74380624	-0.14425571	1.51524442	0.83383834	-0.3127676	2.213924372
miR-340	0.10376824	0.15485045	1.036041799	1.9636503	0.70344645	2.395295836
miR-342	-0.6956859	-0.4977036	1.147092951	0.72641754	-0.37155914	2.140542796
miR-368	-1.0253527	-0.8744446	1.110268107	0.43047336	0.06985306	1.283977837
miR-370	-0.5767027	-0.15929765	1.335523211	1.5487316	0.9104518	1.55647219
miR-373	-1.3240473	-0.5654752	1.69181533	1.2304918	0.08024657	2.219516187
miR-374	-0.70737565	-0.3411978	1.288933516	0.6264192	-0.5052106	2.191061228
let-7b	-0.4348324	-0.35196805	1.059118746	-2.0876024	-1.494535	0.662931908
let-7d	-0.7353358	-0.5639142	1.126167639	-0.4312295	-0.5172812	1.061461246
let-7e	-0.14245269	-0.56296116	0.747161245	0.7613426	0.35227892	1.327823767
let-7g	-0.8111194	-0.5136633	1.228975453	-1.5091286	-0.9442064	0.67599187
let-7i	-0.7547416	-0.47004092	1.218157503	-1.8996499	-1.0022057	0.53683692

Protocolos

EXTRACCIÓN DE RNA TOTAL: *TRIPURE de Roche*

REACTIVOS NECESARIOS:

Cloroformo

Glicógeno

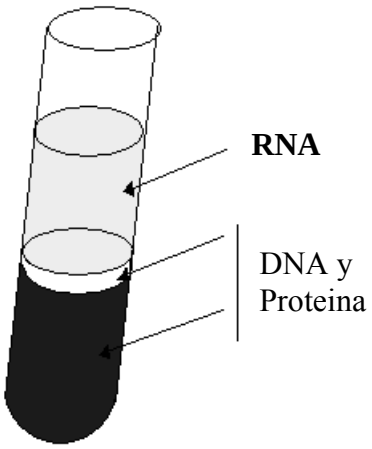
Isopropanol

Etanol al 75%

H2O libre de RNasas (H2O DEPC)

TEJIDO:

1. Para cada 50-100mg de tejido añadir 1ml de TriPure (<10mg añadir 0.8ml TriPure + 5-10µg glicógeno).
2. Homogenizar el tejido con el TriPure (**TRABAJAR EN FRIO).**
3. (Opcional: Centrifugar 12.000 x g 10min a 2-8º C. Quitar la capa de grasa si aparece y transferir el sobrenadante a un tubo nuevo.)
4. Incubar 5min a +15-25º C.
5. Añadir 0.2ml de cloroformo por cada ml de TriPure.
6. Agitar fuerte durante 15s.
7. Incubar el tubo de 2-15min a +15-25º C.
8. Para separar en tres fases: Centrifugar el tubo a 12.000g 15min a +2-8º C.



9. Aislar el RNA de la fase acuosa incolora superior en un eppendorf nuevo de 1,5ml.
10. Precipitar el RNA: Añadir 0.5ml de Isopropanol por cada ml de TriPure.
11. Invertir el tubo varias veces para mezclarlo.

12. Incubar la muestra de 5-10min a 15-25º C (Dejándolo toda la noche a -40º C se obtiene más precipitado).
13. Centrifugar la muestra a 12.000g por 10 minutos a +2-8º C.
14. Descartar el sobrenadante.
15. Añadir 1ml de Etanol 75% por cada ml de TriPure. Vortear.
16. Centrifugar la muestra a 7500 x g 5min a 2-8º C.
17. Descartar el sobrenadante.
18. Dejar secar al aire el exceso de etanol.
19. Resuspender el pellet de RNA en 60-70µl (dependiendo del pellet RNA) de agua libre de RNasas.
20. Pipetear para disolver el pellet de RNA.
21. Incubar 10-15min a 55-60º C.

EXTRACCIÓN DE RNA DE MUESTRAS INCLUIDAS EN PARAFINA: RecoverAll Total Nucleic Acid Isolation (Ambion)

DESPARAFINACIÓN

1. Añadir 1 ml de xilol y vortear.
2. Incubar 3min a 50ºC.
3. Centrifugar 2min a máxima velocidad (si no hay pellet centrifugar 2min más).
4. Eliminar el sobrenadante.
5. Lavar el pellet con 1 ml de etanol 100% y vortexar.
6. Centrifugar a 14.000rpm 2'.
7. Eliminar el sobrenadante.

} x2

8. Incubar durante 10-15min con el tubo abierto.

DIGESTIÓN CON PROTEASA

9. Añadir 400µl de *Digestión Buffer*.
10. Añadir 4µl de proteasa y vortear.
11. Incubar durante 3 horas en el baño a 50ºC.

AISLAMIENTO DE ÁCIDOS NUCLÉICOS (Subir la temperatura del baño a 95ºC)

12. Pasarlo a un tubo de 2ml.
13. Añadir 480µl de *Isolation Additive* y vortear.
14. Añadir 1.1 ml de etanol 100% y pipetear cuidadosamente.
15. Poner 700µl de la muestra en una columna.
16. Centrifugar a 10.000rpm durante 30-60 segundos.
17. Descartar el líquido del tubo colector.

} Hasta finalizar la muestra

18. Lavado con 700µl de *Wash 1*.
19. Centrifugar a 10.000rpm durante 30 segundos.
20. Descartar el líquido del tubo colector.
21. Lavar con 500µl de *Wash 2/3*.
22. Centrifugar a 10.000rpm durante 30 segundos.
23. Descartar el líquido del tubo colector.

24. Centrifugar la columna vacía a 10.000rpm durante 30 segundos.
25. Pasar la columna a un tubo nuevo.

DIGESTION CON LA NUCLEASA Y PURIFICACIÓN

(Precalentar el H_2O DEPC a 95 ºC)

26. Preparar master para tantas muestras como haya:
    - 6µl 10X Dnasa Buffer
    - 4µlDnasa
    - 50µl Nucleasa-free water
27. Añadir 60µl de la master a la columna.
28. Dejar incubar 30 minutos a temperatura ambiente.
29. Añadir 700µl de Wash 1.
30. Incubar 30-60s a temperatura ambiente.
31. Centrifugar 30s a 10.000rpm.
32. Descartar el líquido del tubo colector.
33. Lavar con 500µl de Wash 2/3.
34. Centrifugar 30s a 10.000rpm
35. Descartar el líquido del tubo colector.

x2

36. Volver a centrifugar 1min a 10.000rpm.
37. Pasar la columna a un eppendorf de 1,5ml
38. Añadir 30µl de Elution Solution (precalentada a 95ºC)
39. Dejar un minuto a temperatura ambiente.
40. Centrifugar un minuto a máxima velocidad.
41. Repetir esta elución otra vez.
42. Descartar la columna.
43. Volumen final de 60µl.

WESTERN BLOT

EXTRACCIÓN PROTEÍNA TEJIDO

1. Triturar muy bien el tejido con el tampón de lisis (80-100µl)
2. Dejar en hielo 30' vorteando cada 5'.
3. Centrifugar a 14.000rpm a 4ºC durante 15min.
4. Recojer el sobrenadante.

CUANTIFICACIÓN

1. Hacer recta Standard con BSA 1mg/ml.

0µl	+ 800µl	H2O
1µl	+ 799µl	H2O
2.5µl	+ 797.5µl	H2O
5µl	+ 795µl	H2O
7.5µl	+ 792.5µl	H2O
10µl	+ 790µl	H2O
15µl	+ 785µl	H2O
20ul	+ 780ul	H2O

2. Añadimos 798µl H2O + 2µl de cada muestra en sus respectivos tubos por duplicado.
3. Preparar un "blanco" con tampón de extracción + H2O. Para eliminar el fondo inespecífico
4. Añadimos a todos los tubitos 200µl de Bradford.
5. Vortear.
6. Dejar 20-30 min a temperatura ambiente.Tapado con papel de plata.
7. Leer absorbancia a 595nm.
8. Poner los valores en el excel para calcular la [ ].

PREPARAR GEL

Geles preparados: *Precast Ready gel* de Biorad al 10% Tris-HCL.

CARGAR GEL

- Tenemos que poner entre 30 y 50 µg de muestra.
- En el momento de preparar las muestras se ha de añadir al tampón de carga DTT en una proporción DTT 1:4 SB5X.

- Antes de cargar las muestras desnaturalitzar 5 min a 96-100ºC
- Cargamos 20µl del marcador de peso molecular y todo el volumen que tenemos de las muestras.

ELECTROFORESIS

Carrera

40mA→2 Gels→60V

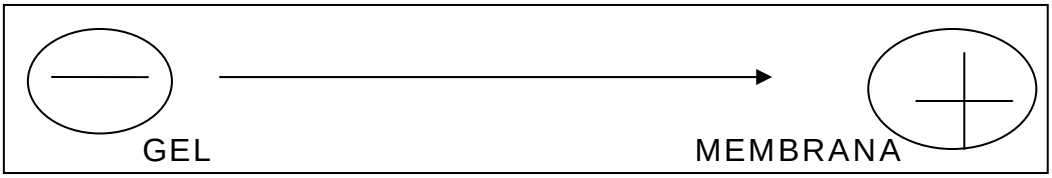
20mA→1Gel

} 2 Horas

- Preparar tampón de transferencia y ponerlo en hielo (ha de estar en frio, nevera).
- Al terminar la carrera poner rapidamente el gel en tampón de transferencia para evitar que difundan las proteínas.

TRANSFERENCIA

- Medir gel (para recortar la membrana a la medida que le toca) y marcar una esquina para saber donde estan las muestras transferidas (utilitzar pinzas).
- Hidratar membrana con H2O.
- Poner con el tampón de transferencia.
- **Remojarlo todo con tampón de transferencia antes de montar.**
- Encima de la membrana poner papel Wattman y pasar rodillo para quitar el aire.
- Ahora pongo el resto del papel.
- Si es posible, hacer la transferencia en frio a 250mA, en la nevera con una mosca y agitador→ O.N.
- Las proteinas corren de:



Chasis	Esponja	3 Papeles Wattman	Gel	Membrana	3 Papeles W	Esponja	Chasis

VIGILAR QUE NO SE FORMEN BURBUJAS ENTRE GEL Y MEMBRANA.

2º DÍA

- Desmontar la transferencia
- Marcar con lapiz las bandas del marcador de peso molecular
- Marcar con un corte la esquina de la membrana
- Con papel de filtro tapar las membranas para que se sequen (pueden estar dias).
- Teñir el gel con Azul de Comassie, para comprobar que haya ido bién la transferencia.
- Rehidratamos las membranes con H2O.

BLOQUEO

- Leche en polvo desnatada disuelta en TBS-T al 5% (5g en 100ml) y poner a agitar en el agitador durante 1h mínimo.

DILUCIÓN ANTICUERPO

- El factor de dilución depende del anticuerpo.
  Diluir en Leche en polvo + TBS-T (5%).
- Incubar O.N.

3r DÍA

- 3 lavados de 10 min con TBS-T, en agitación.
- Preparamos anticuerpo secundario marcado con peroxidasa alcalina, diluido también con leche en polvo + TBS-T (5%).
- Hacer bolsitas con el anticuerpo secundario (Sigma anti mouse peroxidasa dilución→1/5000) y en agitación 1-2 horas.
- Hacer 2 lavados con TBS-T de 10 min cada uno.
- Hacer 1 lavado de TBS 10 min (aquí lo podemos tener bastante rato).

REVELADO Y CUANTIFICACIÓN

Aparato : Chemidoc de Biorad.

Reactivo de Revelado : *Super Signal West Pico Chemiluminescent Substrate*. PIERCE Prod # 34080

- 2 Tampones: Ponemos 1.5 ml de cada tampón
- Mezclarlo
- Secar membranas con papel
- Ponerlas en cubeta con el buffer de revelado, remojar que queden bién cubiertas→1min.
- Volvemos a secar las membranas (entre 2 papeles de filtro)
- Introducir en el aparato.
- Focusing→para mirar si están bién colocadas.
- Fotos cada 10 segundos→ START

TAMPONES NECESARIOS PARA EL WESTERN

TAMPÓN DE CÀRGA SB5X

Para un volumen final de 10ml:

- Sacarosa (50%)-------- 5g
- Tris Base(50mM) -------- 60mg
- EDTA (5mM) ---------- 0.1mL (500mM)
- SDS 5% ----------2.5mL (20%)
- Azul de Bromofenol 0.005% ----500ug

Alicuotar en un eppendorf de 1mL y guardar a -20ºC.
En el momento de preparar las muestras se tiene que añadir DTT (2M) en una proporción 1:4.

TAMPÓN DE LISIS (Tampón TRIS-TRITÁ)

- TRIS HCl pH 7.5 (20mM) ----------0.1ml
- NaCl (150mM)----------0.15ml
- TRITÓ 1%----------0.05ml
- Inhibidor de Proteasas ---------- 0.05ml (No poner hasta el momento de uso)

TAMPÓN DE ELECTROFORESIS

Para un volumen final de 1 litro:

- TRIS BASE (25mM)----------3.03g
- Glicina (192mM)----------14.4g
- SDS 0.1%----------5ml (20%)

Añadir H2O bidestilada hasta 1 litro.

TAMPÓN DE TRANSFERENCIA 10X

Para un volumen final de 1 litro:

- TRIS BASE (250Mm)----------30.285gr
- Glicina (1.92M) ----------144.134gr

Añadir H2O bidestilada hasta 1 litro.

TAMPÓN DE TRANSFERENCIA 1X

Para un volumen final de 1 litro:

- Tampón de transferencia 10X (Biorad)--------100ml
- Metanol ----------200ml

Añadir H2O bidestilada hasta 1 litro.

Agitar antes de poner a la nevera para eliminar el gas que genera el metanol.

NOTA: Recordar no mezclar directamente el tampón de transferencia 10X y el Metanol, sino el tampón precipita y cuesta más de disolverse.

TBS

Para un volumen final de un litro:

- TRIS HCl pH 7.5 (1M) ----------20ml
- NaCl (5M) ----------30ml

Añadir H2O bidestilada hasta 1 litro.

TBST

- TBS + 0.05% Tween-20

TRANSFECCIÓN Y ANÁLISIS EFICIENCIA TRANSFECCIÓN

1. Haremos transfección con siFAM, y lo pasaremos por el citómetro para determinar cuantas células lo han incorporado, con nuestro método de trasfección.

FAM labelled antiMIR negative control 5nM AM-17012

2. Noche antes plaqueamos **SIN ANTIBIÓTICO** en placa de 6 pocillos 250.000 células para que se adhieran.

CALCULO CANTIDADES TRANSFECCIÓN PARA PLACAS DE 6 POCILLOS

Los Pre/Anti miRNA llegan liofilizados a 5Nm +100µl de H2O →stock a 50µM

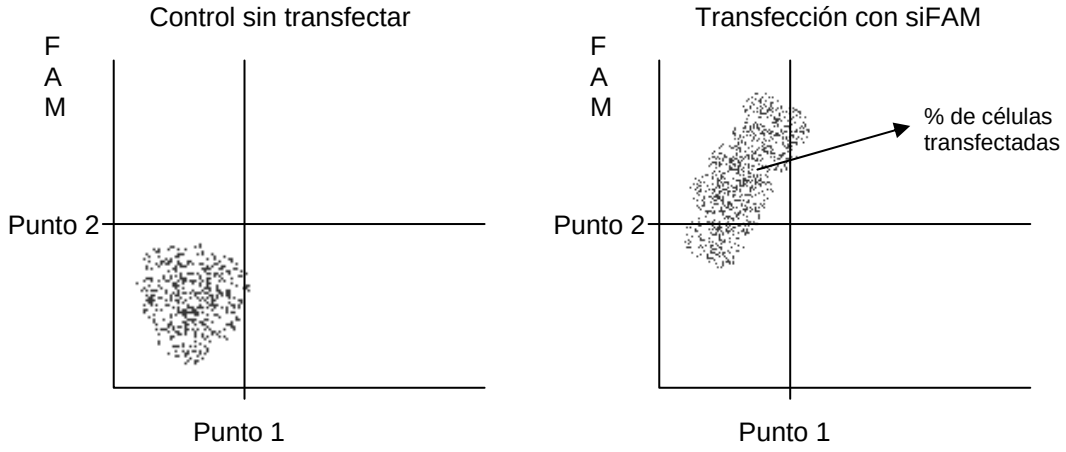
De este stock pondremos 1µl x cada 1ml de Master (Concentración final 50mM).

La lipofectamina*(mantener en hielo) se tiene que poner con su medio→ OPTIMEM*

1ml por pocillo →
- 500µl del Medio de la línea sin Ab **(1)**
- 500µl →
  - 250µl de OPTIMEM + Lipofectamina (5µl/well) **(2)**
  - 250µl de OPTIMEM + MICRO (1µl) **(3)**

1. Dispensamos (1) a cada pocillo
2. Preparamos (2), y lo dejamos 5 minutos a temperatura ambiente.
3. Preparamos (3)
4. Pasados los 5 minutos, añadimos (2) a (3), y mezclamos.

5. Dejar 20 minutos a temperatura ambiente [(2)+(3)] antes de añadir a cada pocillo 500µl. **Dispensar la master de lipofectamina gotita a gotita muy lentamente.** Remover placas con cuidado (10veces por lado).
6. Una vez realizada la transfección, incubar 4 horas en estufa.
7. Pasadas las 4 horas, TRIPSINIZAR.
8. Una vez tripsinizado le añadimos PBS al pellet obtenido (volumen dependiendo pellet, unos 500µl).
9. Pipeteamos para disolver el pellet.
10. Medir en el citómetro.



*** LIPOFECTAMINE 2000**

INVITROGEN

REF. 11668-027 (Size: 0.75ml)

REF. 11668-019 (Size 1.5ml)

REF. 11668-500 (Size 15ml)

*** OPTIMEM**

INVITROGEN REF. 31985

MTS PROTOCOL: *CellTiter 96 AQueous One Solution Cell Proliferation Assay*

Método colorimétrico para la determinación del número de células viables en ensayos de proliferación o de citotoxicidad.

CellTiter 96 AQueous One Solution contiene 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS y un reactivo captador de electrones, phenazine ethosulfate; PES.

El MTS es bioreducido por las células a un componente de color, el formazan, que es soluble en el medio de cultivo. Esta conversión la realiza los NADPH o NADH producidos por las enzimas deshidrogenasas de las células metabólicamente activas.

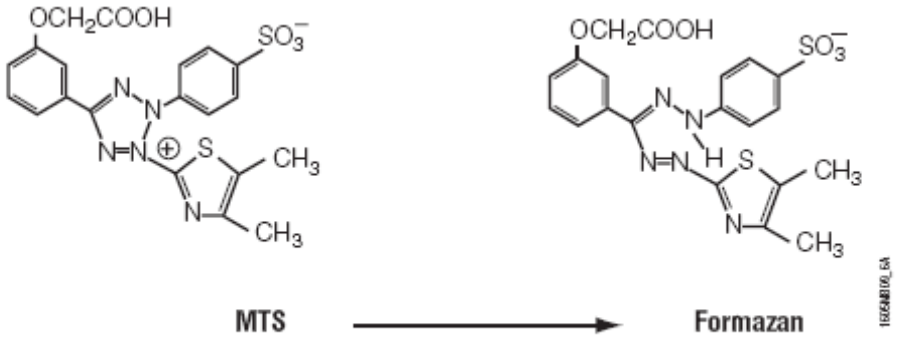
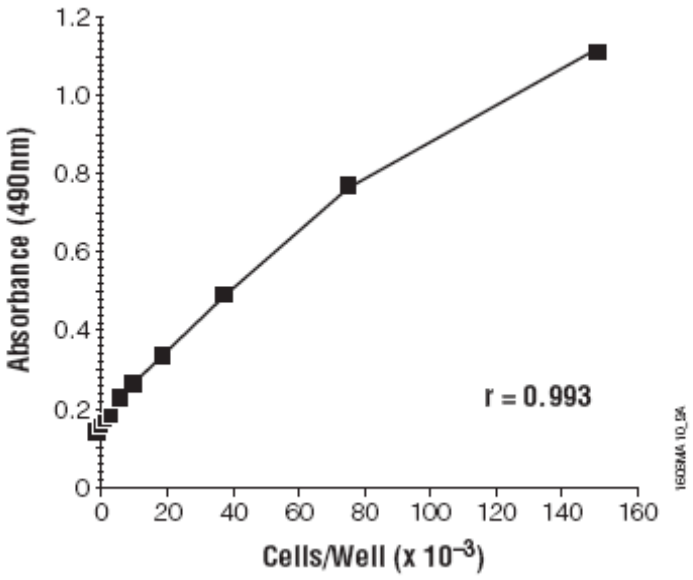


Figure 1. Structures of MTS tetrazolium and its formazan product.

1. Dispensar 1000 cls/pocillo y 2000 cls/pocillo (Se prepara dilución para pipetear en todos los pocillos lo mismo: 50µl o 100µl + la misma cantidad de medio).
2. Dispensar una fila de la placa únicamente con medio para hacer el Blanco.
3. En todos los pocillos de alrededor de las filas a analizar llenar con 100µl de medio para evitar evaporaciones.
4. Poner el MTS a 37ºC mínimo 15 minutos antes de dispensarlo.
5. Cambiar el medio a todos los pocillos antes de dispensar el MTS.
6. Dispensar 20µl de MTS (sin quitar los 100µl que habían de medio). Vigilar no hacer burbujas.
7. Incubar en la estufa 2 horas.

8. Leer Absorbancia a 490nm con una placa de lectura de 96 pocillos. La cantidad de Formazan producido es directamente proporcional al número de células vivas en cultivo.



PROTOCOLO DE TINCIÓN CON CRISTAL VIOLETA

1. Lavar las células con PBS
2. Fijar las células con GLUTARALDEHIDO 0.5% en PBS durante 20 minutos a temperatura ambiente (500µl por pocillo, que recubra).
3. Lavar 3 veces con PBS.
4. Añadir cristal violeta hasta cubrir el pocillo (500µl) y se deja 30 minutos a temperatura ambiente.

> 0.1% disolver en metanol (20 vol) y H2O (80 vol)*
>
> *Se debe trabajar en la campana de sustancias químicas. El cristal violeta se recicla y no se debe tirar por la fregadera.
> REF. Crystal Violet, ACS reagent, anhydrous dye ≥ 90%, SIGMA C6158-50G Batch#:016k3691

5. Lavar con H2O del grifo y dejar secar boca abajo.
6. Para medir en el lector de Elisa: recoger el sobrenadante levantando las células con ácido acético al 10% en PBS, se realiza durante 5 minutos a temperatura ambiente y con 500µl/pocillo y se pone en una placa de ELISA, y se lee a 570nm.

ANEXO 2

Trabajos realizados en el periodo de tesis

Publicaciones a las que ha dado lugar la tesis

blood

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MicroRNA expression profiling in classical Hodgkin lymphoma

Alfons Navarro, Anna Gaya, Antonio Martinez, Alvaro Urbano-Ispizua, Aina Pons, Olga Balague, Bernat Gel, Pau Abrisqueta, Armando Lopez-Guillermo, Rosa Artells, Emili Montserrat and Mariano Monzo

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MicroRNA expression profiling in classical Hodgkin lymphoma

Alfons Navarro,[1] Anna Gaya,[2] Antonio Martinez,[3] Alvaro Urbano-Ispizua,[2] Aina Pons,[1] Olga Balagué,[3] Bernat Gel,[4] Pau Abrisqueta,[2] Armando Lopez-Guillermo,[2] Rosa Artells,[1] Emili Montserrat,[2] and Mariano Monzo[1]

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MicroRNAs (miRNAs) are negative regulators of gene expression that play an important role in hematopoiesis and tumorigenesis. We analyzed miRNA expression in classical Hodgkin lymphoma (cHL) and the influence of Epstein-Barr virus (EBV) infection on the miRNA expression profiles. The expression of 157 miRNAs in lymph nodes from 49 cHL patients and 10 reactive lymph nodes (RLNs) was analyzed by real-time polymerase chain reaction (PCR). Hierarchic clustering revealed 3 well-defined groups: nodular sclerosis cHL, mixed cellularity cHL, and RLNs. A distinctive signature of 25 miRNAs differentiated cHL from RLNs, and 36 miRNAs were differentially expressed in the nodular sclerosis and mixed cellularity subtypes. These results were validated in a set of 30 cHLs and 5 RLNs and in 3 cHL cell lines. mir-96, mir-128a, and mir-128b were selectively down-regulated in cHL with EBV. Our findings suggest that miRNAs play an important role in the biology of cHL and may be useful in developing therapies targeting miRNAs. (Blood. 2008; 111:000-000)

Introduction

Mature microRNAs (miRNAs) are naturally occurring small noncoding RNAs that act as negative regulators of gene expression through messenger RNA interference. These molecules were described for the first time in 1993 by Ambros and colleagues in *Caenorhabditis elegans* (Lee et al[1]), and to date, hundreds of miRNAs have been identified in other species, including viruses.[2,3] miRNAs are encoded by intronic or intergenic DNA regions, primarily as large molecules that can exceed 1 Kb, and are cleaved by an RNase complex into fragments with characteristic stem-loop structures. In the cytoplasm, a RNase called Dicer further cleaves miRNA to generate a duplex molecule of 21 to 25 nucleotides in length.[4] One of the 2 chains is the mature miRNA that binds a protein complex called the RNA-induced silencing complex (RISC). When a miRNA and a messenger RNA exhibit total complementarities, RISC is capable of degrading target messenger RNA,[4] whereas if an incomplete base pairing complementarity takes place, translational silencing of the target occurs. Through these mechanisms, miRNAs decrease translation of human genes.[5,6]

miRNAs play an important role in cellular proliferation and differentiation and embryonic development, and they also act as oncogenes or tumor suppressor genes.[7–10] Notably, the majority of miRNAs are found in cancer-associated genomic regions or in chromosome-fragile sites,[11] suggesting an important role for miRNAs in human tumorigenesis. There is also evidence that the influence of miRNAs in oncogenesis might be indirectly driven. For example, the presence of some viruses in a cell may change the host miRNA pattern.[12] Viruses may participate in the origin of some tumors, such as the Epstein-Barr virus (EBV) in Hodgkin lymphoma (HL).

HL is a neoplasm characterized by the presence of relatively few tumoral cells (Hodgkin and Reed-Sternberg cells) in a nonneoplastic microenvironment.[13] Hodgkin and Reed-Sternberg cells arise from germinal center B cells.[14] Classical HL (cHL) is subclassified according to the morphology of Reed-Sternberg cells and the composition of the cellular background into nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte depletion.[15] The 2 former subtypes are the most frequent forms of cHL and contain a variable proportion of neoplastic cells.

EBV is present in the malignant cells of 40% to 60% of cHL patients. However, the precise role of the EBV in the pathogenesis of cHL is unknown. It has been reported that viruses have their own miRNA set,[16] and that there is an interaction between the host miRNAs and virus miRNAs.[17,18] The interaction between the virus and the malignant cells in cHL might be mediated in part by miRNAs.

To investigate whether a specific expression signature of miRNAs is associated with cHL, we assessed the expression of 156 miRNAs, the majority of which are related to hematopoiesis or tumorigenesis,[7,8,11] in lymph nodes from patients with nodular sclerosis and mixed cellularity cHL and compared the expression patterns with those in reactive lymph nodes (RLNs). We also examined the influence of EBV on the expression pattern of miRNAs in cHL patients.

Methods

Approval for these studies was obtained from the Institutional Review Board of Hospital Clinic, Barcelona. Informed consent was provided according to the Declaration of Helsinki.

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Table 1. Main clinical characteristics of cHL patients, N = 49

Characteristic	No.	%
Median age (range), y	32 (15–80)	
Sex, M/F	27/22	55/45
Histology		
Nodular sclerosis	37	76
Mixed cellularity	12	24
EBV+*	16	36
B symptoms	25	51
Bulky mass	11	22
Anemia, Hb level less than 10^5 g/L	12	24
Leukocytosis, more than 15 × 10^9/L	9	18
Lymphocytopenia, less than 0.6 × 10⁹/L or less than 8% of WBC	7	14
Hypoalbuminemia, less than 40 g/L	17	36
High LDH level, more than 450 UI/L	18	37
High β-2-microblobulin level, more than 2.5 mg/dL	12	29
Stage III-IV	23	47
Hasenclever index†	12	33

WBC indicates white blood cell.

*Eight (26%) of 35 nodular sclerosis versus 7 (70%) of 10 mixed cellularity ($P = .01$).

†Calculated only in 36 patients with advanced disease (stages III-IV, B symptoms, or bulky mass).

Tissue samples and cell lines

Forty-nine specimens of formalin-fixed paraffin-embedded lymph nodes from patients diagnosed with cHL (37 nodular sclerosis and 12 mixed cellularity) between January 1996 and June 2005 were assessed. All patients were diagnosed and followed up in a single institution. Table 1 shows the clinical and biologic characteristics of the patients. Ten RLNs were used as controls. In addition, a validation set of 30 formalin-fixed paraffin-embedded lymph nodes from cHL patients (22 nodular sclerosis and 8 mixed cellularity) and 5 RLNs was analyzed. Finally, human HL cell lines L-428 and HD-MY-Z (nodular sclerosis) and L-1236 (mixed cellularity) were analyzed to discriminate between miRNAs expressed in the cell and those present only in the microenvironment. The cell lines were cultured in RPMI 1640 containing 20% fetal calf serum (Invitrogen, Paisley, United Kingdom).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections, as previously described.[19] Briefly, paraffin sections on silane-coated slides were dewaxed and subjected to antigen retrieval (Target Retrieval Solution; Dako, Glostrup, Denmark) in a microwavable pressure cooker. Primary antibodies against CD20 (L26; Dako), CD5 (NCL-CD5; Novocastra, Dossenheim, Germany), CD30 (BerH2; Dako), and CD15 (Leu M1; Novocastra) were incubated, and the slides were counterstained in Gill hematoxylin and mounted in Pertex (Histolab, Göteborg, Germany).

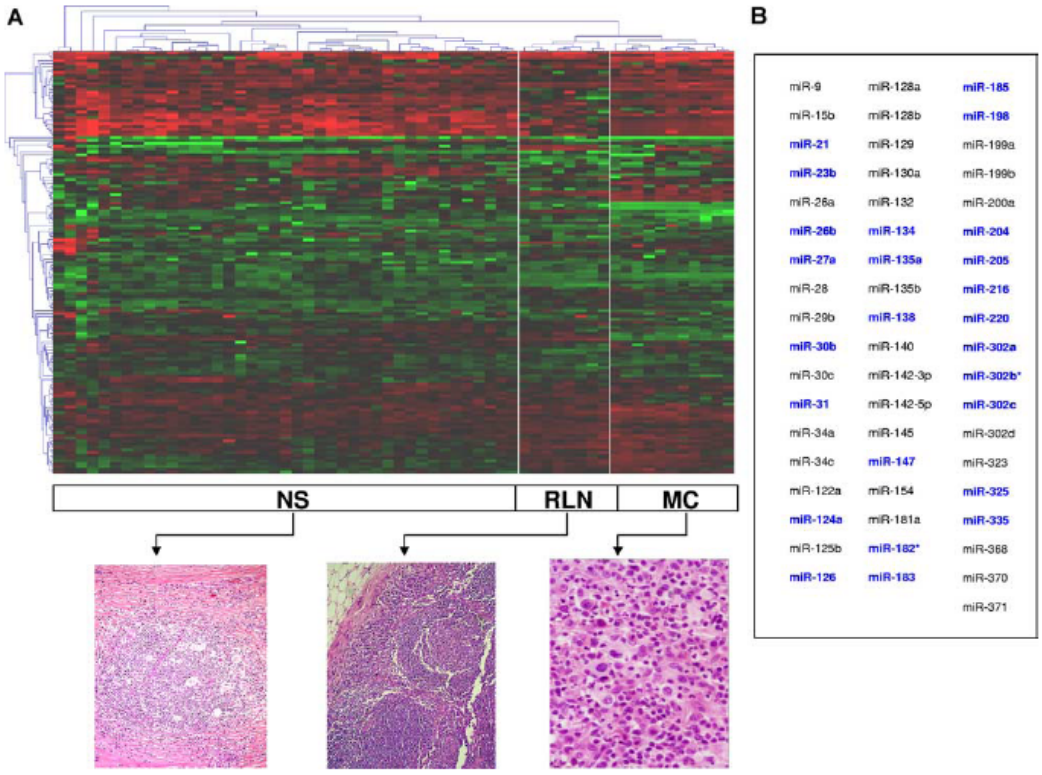

Figure 1. miRNA expression pattern in cHL. (A) Unsupervised hierarchic cluster analysis categorized 3 clusters corresponding to nodular sclerosis (NS), mixed cellularity (MC), and reactive lymph nodes (RLNs). A corresponding typical histology is shown. The data were presented as $\log_{10}$ of relative quantification normalized in regard to global median and relative to the 10 reactive lymph node median as calibration method. (B) A set of 55 miRNAs classified all samples into NS, MC, or RLN (PAM analysis). In blue, the 25 miRNAs comprising the miRNA expression signature capable of distinguishing between cHLs and RLNs.

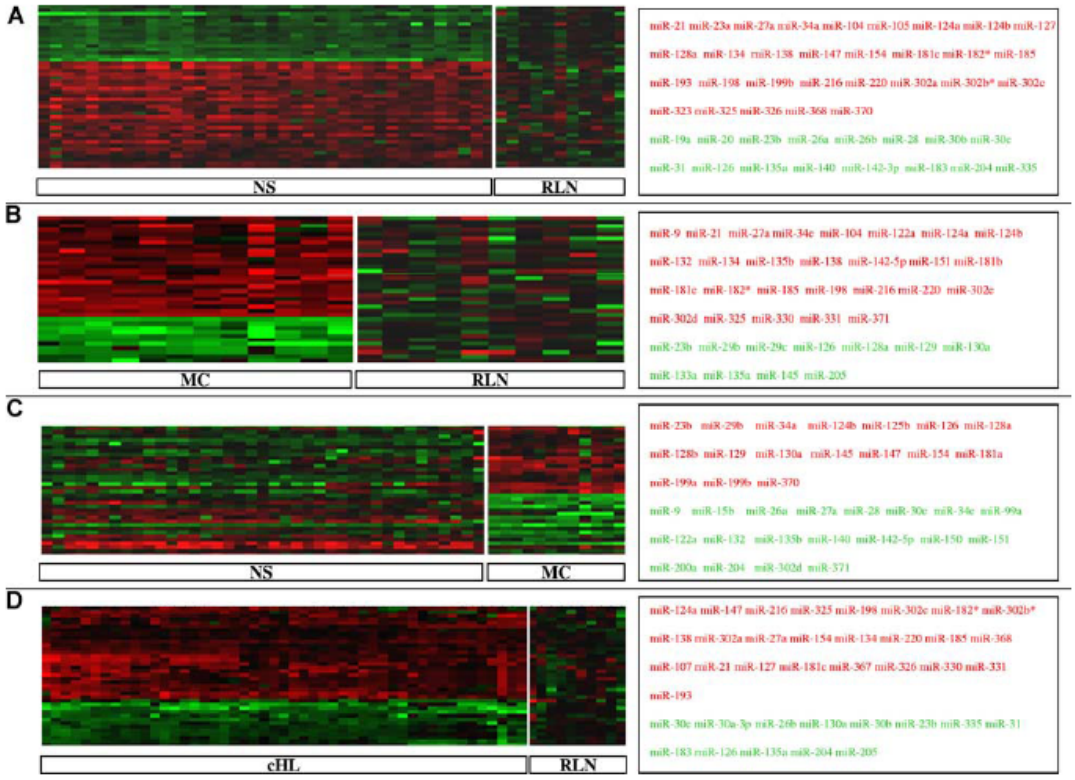

Figure 2. Differential expression of miRNAs in cHL subtypes and in reactive lymph nodes. (A) Nodular sclerosis (NS) versus reactive lymph nodes (RLN). (B) Mixed cellularity (MC) versus reactive lymph nodes. (C) NS versus MC. (D) All cHL cases (NS and MC) versus RLN. miRNAs overexpressed (in red) or underexpressed (in green) are shown in boxes (SAM analysis).

RNA extraction, reverse transcription, and real-time polymerase chain reaction (PCR) quantification

Total RNA was extracted from 49 formalin-fixed paraffin-embedded cHL lymph nodes and from 10 RLNs, using RecoverAll Total Nucleic Acid Isolation (Ambion, Applied Biosystems, Foster City, CA) as per the manufacturer's protocol. The same methods were used for RNA extraction in the validation data set of 30 cHL lymph nodes and 5 RLNs. RNA was extracted from the 3 cell lines using Trizol total RNA isolation reagent (Gibco, Life Technologies, Gaithersburg, MD) as per the manufacturer's protocol.

cDNA was synthesized from total RNA using gene-specific primers of 156 different mature miRNAs (TaqMan MicroRNA Assay Protocol, Early Access Kit; Applied Biosystems) (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Real-time PCR was performed using an Applied Biosystems 7500 Sequence detection system.

miRNA analysis by chromogenic in situ hybridization

Fluorescein (FITC) 5′-labeled locked-nuclei-acid–incorporated (LNA) miRNA ribo probes for mir-21, mir-134, mir-138, and mir-155 (miRCURY LNA detection; Exiqon, Woburn, MA) were used in formalin-fixed, paraffin-embedded tissue sections on silane-coated slides (Vision BioSystem, Mount Beberly, AU). Chromogenic in situ hybridization was performed in an automated platform Bond Max (Vision BioSystems). Slides were pretreated with protease 1 for 10 minutes at 37°C. A total amount of 300 μL 25-nM probe was hybridized in 1 × sodium chloride–sodium citrate hybridization buffer (SSC) (Innogenetics, Antwerp, Belgium) up to 50°C for 2 hours. We used a prediluted mouse anti-FITC antibody (Vision BioSystems) for 20 to 60 minutes followed by a biotin-free, polymeric horseradish peroxidase (HRP)–linker antibody conjugate system (Refine Detection System; Vision BioSystems). DAB was used as a chromogen reacting for 10 minutes and hematoxylin was used as a counterstain.

EBV analysis

The presence of EBV in cHL lymph nodes was examined by in situ hybridization for EBV RNA in an automated platform BenchMark XT (EBER 1 and 2, Inform EBER; Ventana Medical Systems, Tucson, AZ), and real-time PCR using specific primers and probe for the highly conserved segment BamH1 W of EBV.[20]

Statistical analysis

miRNA expression data were normalized by 2 different approaches: global-median normalization and let7-a miRNA as previously described.[21] Data were analyzed using BRB Array Tools version 3.5.0 software and TIGR Multiexperiment viewer version 4.0 software. Hierarchic clustering was performed using average linkage and Euclidean distance. To identify miRNAs with significant differential expression between the 2 histologic subgroups and those that might be influenced by the presence of EBV, 2 multivariate permutation tests were performed: significance analysis of microarrays (SAM) and Student *t* test based on multivariate permutation (with random variance model). Differences between miRNAs were considered statistically significant if the *P* value was less than .001. The prediction analysis of microarrays (PAM) and class prediction methods (BRB Array Tools) were used to determine a set of miRNAs able to classify the samples into cHL and RLNs and to differentiate between the 2 histologic subgroups. To identify functional interactions of putative target genes of miRNAs,

Table 2. Composition of the miRNA signature of cHL

miRNA	Chromosomal location	Expression level	Putative targets*
miR-21	17q23.2	High	*PTEN,† TPM1,† TRAIL-3,*
miR-23b	9q22.32	Low	*NOTCH1,† SUMO1, PLK3, POU4F2*
miR-26b	2q35	Low	*MMP21, IFNG*
miR-27a	19p13.12	High	*CD44*
miR-30b	8q24.22	Low	*CCNE1, ITGB3, ITGA5, TIMP-2, TIMP-3, SERPINE1*
miR-31	9p21.3	Low	*CD28, CD48, EBF3, TRAF3*
miR-124a	8p23.1	High	*ITGB1,† ANGPT1†*
miR-126	9q34.3	Low	*CD97, BAD, IKBKAP, VCAM1, TNFC, TNFS11, PIK3C*
miR-134	14q32.31	High	*J-CHAIN*
miR-135a	3p21.2	Low	*MSH2*
miR-138	7q32.2	High	*PU.1, TCF3, E2A, FAK, HIF-1A*
miR-147	9q32.2	High	*NOL3, ZAP-70*
miR-182*	7q32.2	High	
miR-183	7q32.2	Low	*ITGB1*
miR-185	22q11.21	High	*PBX1, CD79B*
miR-198	3q13.33	High	*CCND2, BCL7A*
miR-204	9q21.13	Low	*ATF2, BCL2, CDC25B, BCL9, BCL11A, BCL11B*
miR-205	1q32.2	Low	*K-RAS, SMAD4, MSH2, PTEN*
miR-216	2p16.1	High	*BCL11B, BCL9*
miR-220	Xq25	High	*IRF3*
miR-302a	4q25	High	*CD45, CD138, RECK, CXCR4*
miR-302b	4q25	High	*CD45, CD138, RECK, CXCR4*
miR-302c	4q25	High	*CD45, CD138, RECK, CXCR4*
miR-325	Xq21.1	High	*NFkB-REPRESOR FACTOR*
miR-335	7q32.2	Low	*ANGPT1*

The putative targets shown were selected based on functional aspects (David Database) and on described gene associations (GENIG).
*Putative target genes identified from Mirbase and TargetScan using DAVID database and GENIG software.
†Target genes experimentally validated were identified from Tarbase.

obtained from Mirbase,[22] TargetScan,[23] and Tarbase[24] databases, we used DAVID database[25] and GENIG (Technical University of Catalonia and CEPBA-IBM Research Institute, http://alggen.lsi.upc.edu).

Results

miRNA patterns in cHL patients and in RLNs

Unsupervised hierarchic clustering of the cHL and RLN samples was performed on the entire set of unfiltered data. The heat map of miRNA expression categorized 3 well-defined clusters corresponding to nodular sclerosis cHL, mixed cellularity cHL, and RLNs (Figure 1). Thirty-eight miRNAs were differentially expressed in cHL versus RLNs (Figure 2; Table S2), and a set of 55 miRNAs was able to further classify all samples into nodular sclerosis cHL, mixed cellularity cHL, or RLNs. (Figure 1). A specific miRNA expression signature consisting of 25 of these 55 miRNAs precisely differentiated between cHLs and RLNs (Figure 1; Table 2). In addition, 36 miRNAs were differentially expressed in nodular sclerosis and mixed cellularity subtypes (Figure 2). These findings were confirmed in the validation set of 30 cHLs and 5 RLNs (Figure S1).

Analysis of miRNA expression in human HL cell lines

cHL tumors are composed of different reactive cell types and tumor cells represent a minority. To elucidate whether different signatures might discover different tumor compositions rather than be specific for tumor cells, we analyzed the 25-miRNA signature that discriminated between cHLs and RLNs in 3 different human HL cell lines: L-428, HD-MY-Z, and L-1236 (Figure 3). We found that 20 of the 25 miRNAs analyzed were expressed by the human HL cell lines and were therefore likely to have been expressed by the tumor cells rather than the microenvironment. The 5 miRNAs (miR-220, miR-302a, miR-302b, miR-302c, and miR-325) that were not expressed in the cell lines, and had yet to be overexpressed in the cHL cases may, have been expressed by the reactive microenvironment. MiR-21, miR-27a, miR-147, miR-182, miR-183, and miR-216 were the most strongly up-regulated miRNAs in the HL cell lines (Figure 3A).

Moreover, we analyzed the 36 miRNAs that were differentially expressed in the nodular sclerosis and mixed cellularity subtypes and found that 32 were expressed in the cell lines; miR-122a, miR-154, miR-302d, and miR-371 were not expressed in the cell lines, suggesting differences in the reactive microenvironment. MiR-34a, miR-128b, miR-129, and miR-200a were the most strongly differentially expressed miRNAs between L-428 and HD-MY-Z nodular sclerosis HL cell lines versus L-1236 mixed cellularity HL cell line.

miRNA analysis by chromogenic in situ hybridization

To examine whether miRNAs that were more highly expressed in cHLs than in RLNs were detected preferentially in tumor or reactive cells, we analyzed 20 cHL lymph nodes using highly sensitive chromogenic in situ hybridization. We first analyzed miR-155 as a positive control, as previously described in cHL.[26] We observed a cytoplasmic signal in Hodgkin and Reed-Sternberg cells, as well as in scattered reactive lymphocytes and activated histiocytes, as recently reported[27] (Figure 4). Based on functional and target analyses and on the chromosomal locations of the miRNAs (Table 2), we selected 3 miRNAs with a potential role in tumorigenesis and analyzed them by chromogenic in situ hybridization in the 20 cases: miR-21 (validated target *PTEN*, encoded in

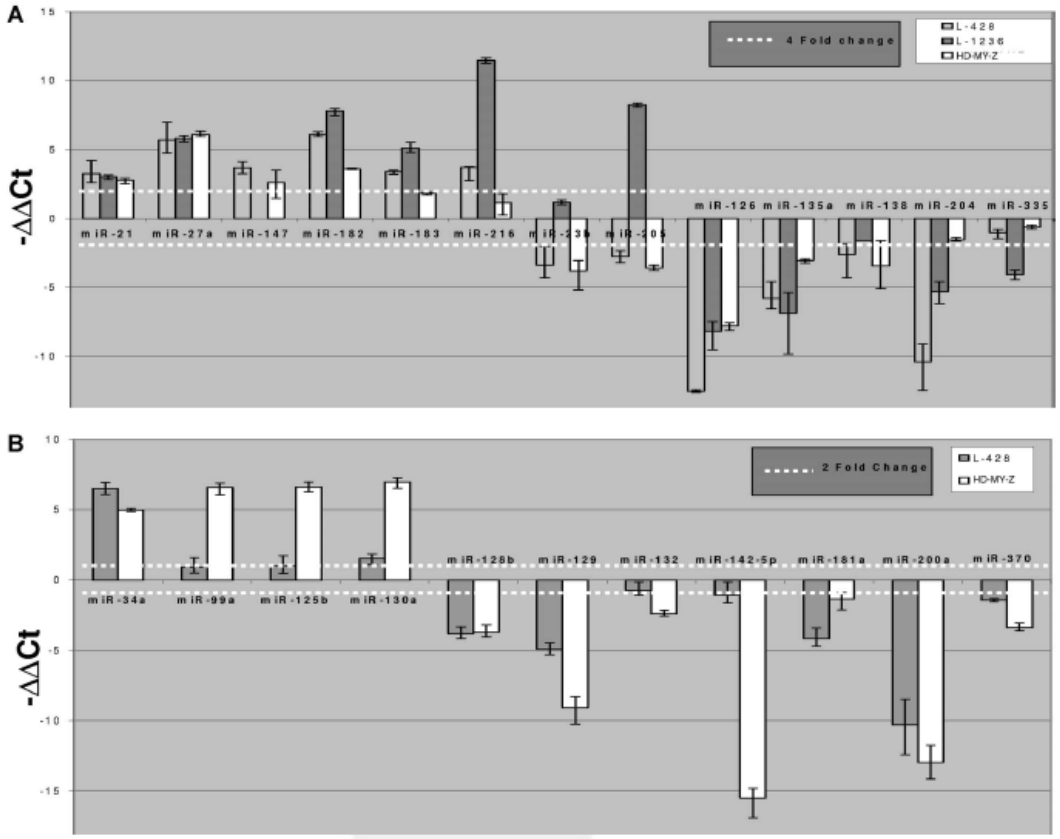

Figure 3. Analysis in human Hodgkin lymphoma (HL) cell lines: L-428 and HD-MY-Z (nodular sclerosis) and L-1236 (mixed cellularity). (A) 25-miRNA signature. This figure shows the $\log_2$ of fold change ($-\Delta\Delta$Ct) of the 13 miRNAs most differently expressed in the cell lines. The dotted line indicates a 4-fold difference in expression compared with the mean of expression of reactive lymph nodes. (B) Thirty-two of the 36 miRNAs differentially expressed in lymph nodes of nodular sclerosis and mixed cellularity subtypes were also differentially expressed in the cell lines. Panel B depicts only the 11 miRNAs with at least a 2-fold difference in expression between the 2 subtypes.

17q32.2[28]); miR-134 (putative target *J-chain,* encoded in 14q32.31[28]); and miR-138 (putative target *PU.1,* encoded in 7q32.2[29]). In all 20 cases, a cytoplasmic signal was observed in Hodgkin and Reed-Sternberg cells, and we also observed a certain degree of miRNA expression in surrounding lymphocytes. Moreover, a nuclear signal was identified in some reactive tumor-infiltrating lymphocytes for miR-21 and miR-138 (Figure 4).

Effect of EBV infection on miRNA expression in cHL

Ten miRNAs were differentially expressed in EBV$^+$ cHL compared with EBV$^-$ cHL. In EBV$^+$ cases, miR-96, miR-128a, miR-128b, miR-129, and miR-205 were underexpressed and miR-28, miR-130b, miR-132, miR-140, and miR-330 were overexpressed (Figure 5). In the subgroup of nodular sclerosis cHL, 3 miRNAs (miR-96, miR-128a, and miR-128b) were significantly underexpressed in EBV$^+$ cHL compared with EBV$^-$ cHL. All but one of the mixed cellularity cHLs were EBV$^+$.

Association between miRNA expression and clinical parameters

We analyzed a possible association of miRNA expression with clinical and biologic patient characteristics: age, sex, B symptoms, bulky mass, anemia, leukocytosis, lymphocytopenia, hypoalbuminemia, ESR, LDH level, β-2-microglobulin level, extranodal involvement, stage, Hasenclever index, positivity for CD15, positivity for CD20, and density of tumoral cells and T lymphocytes in the tumor microenvironment. We found that miR-138 was overexpressed in Ann-Arbor stage I-II disease ($P = .003$), while miR-328 was overexpressed in Ann-Arbor stage III-IV disease ($P = .004$). To further investigate this possible association between miRNA expression and disease stage, we analyzed the expression of miR-138 and miR-328 in 30 additional patients from the validation data set (18 stage I-II; 12 stage III-IV). When results for both the original set and the validation set were combined, we found that miR-138 was overexpressed in stage I-II disease but not in stage III-IV ($P = .001$) (Figure S2), but no association was observed between miR-328 expression and disease stage.

Discussion

Prior studies have shown that a small subset of miRNAs may define tumor entities better than microarray expression data from thousands of messenger RNAs.[30] In the present study, we have

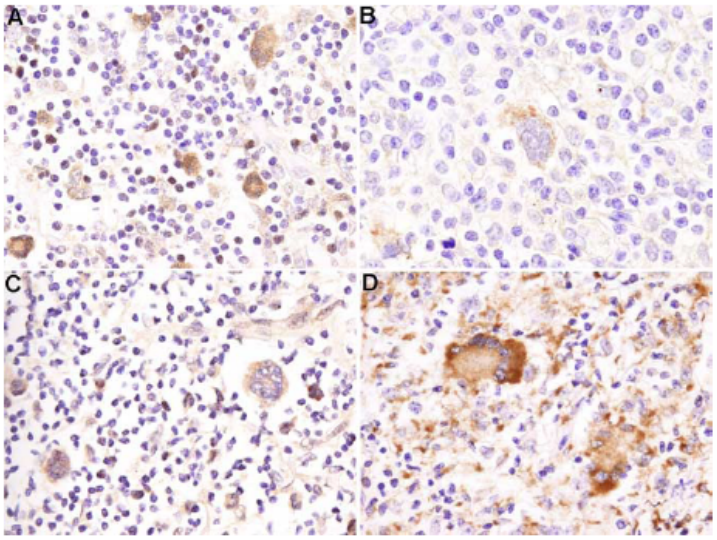

Figure 4. Chromogenic in situ hybridization of cHL cases. Cytoplasmatic expression in Hodgkin and Reed-Sternberg cells of miR-21 (A), miR-134 (B), miR-138 (C), and miR-155 (D). miR-155 was used as positive control of the hybridization technique. (Microscope, Olympus BX51 [Olympus, Center Valley, PA]; camera, Olympus DP70; lens, UPlanFl 40 ×/0.75; software, Olympus DP Controller.)

characterized for the first time a 25-miRNA signature that can differentiate between cHLs and RLNs. In addition, a small number of miRNAs were differentially expressed in mixed cellularity and nodular sclerosis subtypes. Finally, overexpression of one miRNA (miR-138) was related with Ann-Arbor stage I-II cHL. miR-138 has been reported to be overexpressed in other tumors,[31] where it seems to be associated with an undifferentiated state.[32]

The differential miRNA expression observed between cHLs and RLNs may be explained by cytogenetic changes in the Hodgkin and Reed-Sternberg cells. The genomic region 17q has previously been associated with frequent gains in cHL,[28] and miR-21 is encoded in this chromosomic region (Table 2). Other previously described chromosomal gains in cHL[28] include 2p, where mirR-216 is encoded, 22q, where miR-185 is encoded, and 14q, where miR-134 is encoded. One of the most frequent losses involves 4q, where miR-302a, miR-302b, and miR-302c are encoded,[28,33] and 3p, where miR-135a is encoded.[34]

The analysis of the 25-miRNAs cHL signature in cell lines showed a set of strongly up-regulated miRNAs (miR-21, miR-27a, miR-147, miR-182, and miR-216) and a set of down-regulated miRNAs (miR-126, miR-135a, and miR-204) supporting our observations in patient samples. However, other miRNAs showed a different expression pattern in the patient samples compared with the cell lines. Those differences could be due to the expression of miRNAs by the reactive microenvironment in patient samples or to the molecular and chromosomic alterations of cell lines produced by the immortalization process.[35]

Chromogenic in situ hybridization showed a preferential expression of miR-21, miR-134, and miR-138 in the cytoplasm of Hodgkin and Reed-Sternberg cells, suggesting that miRNA silencing may be biologically relevant in cHL tumor cells; moreover, the miRNA expression observed in lymphocytes provides some evidence that miRNA expression in the tumor microenvironment is also important to the biology and clinical behavior of cHL.[13,36,37] The cHL tumor microenvironment differs among the histologic subtypes, and in the present study, a differential pattern of miRNA expression was observed in mixed cellularity and nodular sclerosis subtypes. The subsequent analysis of cell lines revealed that some

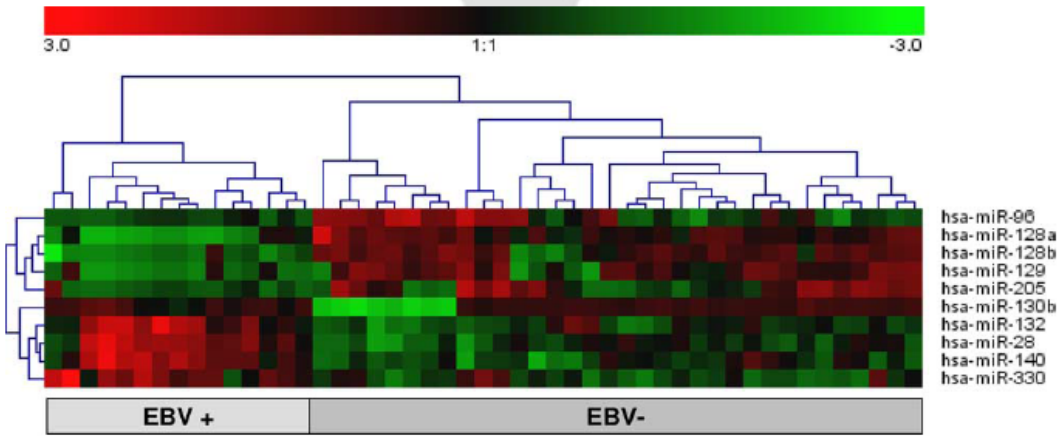

Figure 5. MiRNAs differentially expressed in EBV⁺ versus EBV⁻ cases.

miRNAs were also differentially expressed in the Hodgkin and Reed-Sternberg cells in the 2 histologic subtypes. Although prior studies had shown that miR-155 is overexpressed in cHL,[26] the molecular signature in the present study does not include miR-155. However, chromogenic in situ hybridization showed that miR-155 was constantly expressed in atypical cells in all the cHL cases analyzed as well as in reactive lymphocytes and activated macrophages.[26,27]

Since miRNAs differentially expressed in cHL cases target putative and validated genes involved in survival, apoptosis, and B-cell functions, our findings can provide the basis for a better understanding of the complex mechanisms of transformation of a normal B cell into a tumor cell. One of the most striking features of cHL tumor cells is their acquisition of survival advantages while largely lacking expression of most of the B-cell–associated genes.[14,38,39] miR-21, which was overexpressed both in cHL lymph nodes and in the human HL cell lines, has been reported to favor cell survival by indirect up-regulation of antiapoptotic genes.[40-44] cHL is a neoplasm of B cells characterized by an incomplete B-cell phenotype, due to down-regulation of some transcription factors crucial to the full development of a mature B-cell program.[14] The mechanisms causing down-regulation of the transcriptional program in cHL cells are not totally understood, but some epigenetic events are proving to be important in the silencing of B-cell genes.[45] miRNAs may be a new regulatory epigenetic event, with an important role regulating the translation of a number of different genes in the complicated regulatory network that leads normal B cells from the germinal center[46] to Hodgkin and Reed-Sternberg cells.

Prior reports had suggested that virus miRNAs can play an important role in the host-pathogen interaction networks. Moreover, viruses might trigger changes in the host miRNA expression pattern, thus favoring cancer development.[12,47,48] We identified a subset of 10 host miRNAs whose expression was influenced by the presence of EBV. The effect of EBV on host miRNAs might explain the reported association of EBV with the clinical course of cHL patients.[49] Interestingly, only one of the miRNAs differentially expressed in EBV^+ cases was included in the 25-miRNA expression signature differentiating cHL from RLN, leading us to speculate that EBV is not a primary transforming event in cHL, a concept that is also supported by the fact that the majority of the cHL cases were EBV^-.[49]

In summary, cHLs express a characteristic miRNA signature different from that of normal RLNs, with a small number of miRNAs differentially expressed in mixed cellularity and nodular sclerosis and one differentially expressed in early- and advanced-stage disease. Some of these miRNAs were expressed in Hodgkin and Reed-Sternberg cells but not in reactive cells. In addition, EBV influences host miRNA expression in cHL. These findings suggest that miRNAs may play an important role in the biology of cHL, and they may be useful in the development of therapies targeting miRNAs in tumor cells in cHL patients.[40,50]

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Authorship

Contribution: A.N. designed and performed the research, analyzed the data, and wrote the paper; A.G. designed the research, selected cases, analyzed the clinical data, and wrote the paper; A.M. designed and performed the research, analyzed the data, and wrote the paper; A.U.-I. and M.M. designed the research, analyzed the data, and wrote the paper; A.P. and O.B. performed the research and analyzed the data; B.G. analyzed the data and performed the statistical analysis; P.A. and A.L.-G. analyzed the clinical data; R.A. analyzed the data; E.M. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Participaciones en congresos relacionadas con el tema de tesis

CONGRESOS INTERNACIONALES

Autores: Alfons Navarro, Anna Gaya, Aina Pons, Pau Abrisqutea, Bernat Gel, Antonio Martinez, Armando Lopez-Guillermo, Carmen Martinez, Miquel Granell, Rosa Artells, Sonia Jansa, Alvaro Urbano-Ispizua, Emili Montserrat, Mariano Monzo.

Título: *Analysis of microRNA patterns in Hodgkin's Lymphoma (HL).*

Tipo de participación: Comunicación oral

Congreso: 48TH ASH Annual Meeting. December 2006

Publicación: Blood

Lugar de celebración: Orlando (USA) **Año:** 2006

Autores: Alfons Navarro, Anna Gaya, Antonio Martinez, Alvaro Urbano-Ispizua, Aina Pons, Olga Balague, Bernat Gel, Pau Abrisqueta, Armando Lopez-Guillermo, Rosa Artells, Emili Montserrat, Mariano Monzo

Título: *Analysis of microRNAs expression pattern in classical Hodgkin lymphoma*

Tipo de participación: Comunicación oral

Congreso: EHA 2007- European Hematology Association

Publicación: Haematologica

Lugar de celebración: Viena (AUSTRIA) **Año:** 2007

Autores: Jesús G. Foncillas, Alfons Navarro, Eva Bandres, Rosa Artells, Isabel Moreno, Bernat Gel, Rafael Ibeas, Jose Moreno, Francisco Martínez, Maribel de Miguel, Mariano Monzo.

Título: *Cluster mir-17-92 is differentially espressed in colon cancer PATIENTS and embryonic colon tissue and may contribute to carcinogenesis through E2F1 expression.*

Tipo de participación: Poster discussion

Congreso: ASCO 2007, American Society of Clinical Oncology

Publicación: Journal of Clinical Oncology

Lugar de celebración: Chicago (USA) **Año:** 2007

Autores: Alfons Navarro, Anna Gaya, Antonio Martinez, Alvaro Urbano-Ispizua, Aina Pons, Olga Balague, Bernat Gel, Pau Abrisqueta, Armando Lopez-Guillermo, Rosa Artells, Emili Montserrat, Mariano Monzo

Título: Mir-135a Expression is associated with relapse in Hodgkin lymphoma

Tipo de participación: Poster

Congreso: ASH 2007, American Society of Hematology

Publicación: Blood

Lugar de celebración: Atlanta (USA) **Año:** 2007

Autores: Alfons Navarro, Antonio Martinez, Olga Balagué, Anna Gaya, Aina Pons, Alvaro Urbano-Ispizua, Emili Montserrat, Mariano Monzo
Título: MicroRNA analysis by in situ hybridization in Hodgkin lymphoma
Tipo de participación: Poster
Congreso: ASH 2007, American Society of Hematology
Publicación: Blood
Lugar de celebración: Atlanta (USA) **Año:** 2007

CONGRESOS NACIONALES

Autores: Alfons Navarro, Anna Gaya, Alvaro Urbano-Ispizua, Aina Pons, Bernat Gel, Miquel Granell, Rosa Artells, Sonia Jansà, Carmen Martinez, Armando Lopez-Guillermo, Emili Montserrat, Mariano Monzo M.
Título: Análisis de la expresión de micro RNA en el linfoma de Hodgkin (LH)
Tipo de participación: Comunicación oral
Congreso: XLVIII REUNION NACIONAL AEHH, XXII CONGRESO NACIONAL SETH
Publicación: Haematologica
Lugar de celebración: Granada **Año:** 2006

Autores: Silvia Pairet, Alfons Navarro, Aina Pons, Anna Gaya, Antonio Martínez, Alvaro Urbano-Ispizua, Mariano Monzó
Título: Estudio de la expresión de miRNAs en diferentes tipos celulares
Tipo de participación: Poster
Congreso: XX Congreso Nacional Asociación Española de Técnicos de Laboratorio
Lugar de celebración: Santander **Año:** 2007
Código: 273761 **Orden:** 015

Otras publicaciones realizadas durante el periodo de tesis

1. Bandres E, Cubedo E, Agirre X, Malumbres R, Zarate R, Ramirez N, Abajo A, **Navarro A**, Moreno I, Monzo M, Garcia-Foncillas J.
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